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METABOLISM AND PHARMACOLOGY OF INORGANIC AND FLUORINE CONTAINING COMPOUNDS

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FOREWORD

This study was initiated by the Biomedical Laboratory of the Aerospace Medical Research Laboratories, Aerospace Medical Division, Wright-Patterson Air Force Base, Ohio, under Project 6302, "Toxic Hazards of Propellants and Materials," Task 630202, "Pharmacology-Biochemistry." The research was performed under Contract No. AF 33 (615)-1799, with the Radiation Center, Oregon State University, Corvallis, Oregon. Dr. C. H. Wang was the Principal Investigator for the Oregon State University, and Dr. K. C. Back, Chief, Toxic Hazards Branch, was Contract Monitor for the Aerospace Medical Research Laboratories. Research was initiated July 1, 1964 and completed June 30, 1967.

The valued technical assistance of John Clevenger, Thurman Cooper, Dean Johnson, Chris Pillsbury, Moti Pinjani and Vernon Smith of the Radiation Center is acknowledged.

This technical report has been reviewed and is approved.

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ABSTRACT

Studies have been made of the toxicology and chemistry of nitrogen trifluoride, tetrafluorohydrazine, chlorine trifluoride, bromine pentafluoride, and oxygen difluoride. Lethality of each agent by inhalation has been determined, as well as estimates of lethality after intraperitoneal administration. Experimental evidence suggests that the lethal effect of interhalogens is by corrosive local destruction of pulmonary surfaces, resulting in failure of gas exchange. Oxygen difluoride is thought to pass intact into the pulmonary cells where it reacts with biochemical reducing systems to ultimately cause cell death and structural failure. The nitrogen fluorides both cause intrinsically lethal levels of methemoglobin formation, but other pharmacologic activity by these compounds or their derivatives may also contribute to their toxic activity.

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SECTION I

GENERAL INTRODUCTION

The evaluation of a prospective missile propellent includes, with various engineering considerations, judgment of the hazards which it might present to any segment of the human or animal population as a result of accidents during production, use, or transportation. As a part of this evaluation, the toxicology of several compounds considered for deployment as oxidizers in missile propulsion has been investigated and is discussed in this report. The compounds, nitrogen trifluoride (NF_3), tetrafluorohydrazine (N_2F_4), chlorine trifluoride (ClF_3), bromine pentafluoride (BrF_5) and oxygen difluoride (OF_2), differ in many physical and chemical properties, but are each relatively simple molecules containing a large percentage of fluoride in highly oxidizing states.

In simplest terms, the initial investigation in this program has been directed toward establishing or confirming the basic information on the symptoms and lethality caused by inhalation of inorganic fluoride oxidizing agents by small animals. The subsequent task was to investigate the site and mechanism of the primary toxic activity. These activities were in turn dependent upon the development of safe, precise methods of handling and administration of these highly reactive compounds, an effort which constituted a major portion of the first phase of the program (ref. 1).

The selection of parameters for identifying toxic manifestations of a compound is influenced to some extent by symptomatology and the degree of lethal response. However, more valuable guidance usually arises from existing studies of compounds having some similarity to the agent in question, and for which at least some specificity has been established. Unfortunately, existing information about the agents under study is limited to lethality data and in some cases, histopathological and hematological observations. With these deficiencies, it became important to first determine simply whether the effects of these compounds are systemic or are due to lesions induced at the site of contact.

By a somewhat arbitrary process, therefore, a number of parameters were selected for assessment by established clinical chemical laboratory assays in the hope of finding indications of systemic toxicity which would then be followed in more detail. To this end, distribution of fluoride in tissues of intoxicated rats has also been studied. At the same time, the basic physical and chemical properties of the compounds were carefully reviewed in order to help in predicting the persistence and

reactions of each agent in the atmosphere, in the environment of the lung, and in the circulation and tissues. It was necessary, as well, to investigate the basic chemistry of several of the compounds in terms of the biological environment they may be expected to contact.

Because of the diffuse nature of the studies under this contract, specific phenomena which appeared worthy of detailed observation were left untouched. Some of the routes of investigation which were ultimately followed have been productive, and many were less well chosen. Some have led to potential studies not germane to the problems set forth under this contract.

In the early portion of the program, many of the decisions to direct attention toward or away from a given investigation were necessarily made with a minimum of information. As the various studies have progressed, it has occasionally become apparent that previously rejected approaches were viable; with greater background knowledge, entirely new ideas meriting study have also emerged. The problem of adopting or rejecting these additional potentially successful researches has become, in the latter part of the contract period, less a matter of decision than of committed time and resources. The result has been that while we have obtained substantial information and understanding of the mechanisms of these toxic compounds, in most cases we have simply laid the groundwork for more detailed and informative work in the future.

SECTION II

METHODS

This section describes methods which are common to the entire program. Procedures which are peculiar to an area of study are described in the appropriate section. Materials, equipment and instrumentation are listed in the appendix.

Control and Utilization of Reactive Gases

The conduct of the entire program described in this report has depended upon precise and safe methods for handling the inorganic fluoride oxidizing agents involved. The procedures we have devised have been described in detail (ref. 1), and in a later report a number of improvements have been noted (ref. 2).

Gases are diluted on the basis of ratios of flow rates. The manifold system is customarily capable of stepwise dilutions to one volume of toxic gas per million volumes of atmosphere, and it can be modified to deliver dilutions of one part per billion. Flow rates are measured directly by mass flowmeters which are calibrated as a function of the relative molar heat capacity of gases. There is no contact between sensing probes and reactive material; as a result, no change in calibration occurs with use. Since the heat capacity of gases is nearly nonresponsive to wide changes in temperature and pressure, regulation of gas flow is greatly simplified by these instruments. Methods of verifying gas dilutions have been described elsewhere (ref. 1).

Many of the problems of studying these reactive gases originate with the materials of construction. At the onset of the program, information available in the literature and from experiments in this laboratory enabled us to find materials suitable to our requirements. Our findings and those of others have been mentioned previously (ref. 1). Since that time, we have found that more latitude is possible with some of the inorganic fluoride compounds, and in other cases, materials thought suitable have been rejected. The most notable improvement has been in the use of polyethylene and similar materials in direct contact with various other agents. 1,500 ppm ClF_3 in air was found essentially unreactive with thin polyethylene bags, which then were successfully used as dynamic exposure chambers for large plants. The good visibility has made them useful in exposure of rats as well. Undiluted gaseous N_2F_4 and NF_3 can be held in polyethylene disposable syringes with neoprene plungers for hours with no detectable change in their infrared

spectra or in their biological effects. A polyethylene bag 0.002 inches thick has been inflated with NF_3 and left as long as two weeks with no detectable reaction. We have also concluded that while copper and brass are excellent materials over short periods of use, repeated changes in the gases used and long periods of contact, particularly with interhalogens, make the use of stainless steel mandatory in both tubing and valve construction.

Exposure Procedures

A small cylindrical exposure chamber 6 inches in diameter and 8 inches long, lined with Teflon was used in most exposures to interhalogens and N_2F_4. These compounds are subject to decomposition in moist air, but by using high flow rates, they can be carried intact through a small chamber with nonmetallic walls even in the presence of animals.

Exposures to relatively stable gases such as NF_3 and OF_2 were made in an 18" x 18" x 27" aluminum chamber lined with Teflon surfaced glass cloth. This chamber is equipped with an air lock which permits rapid transfer of rats into and from the chamber without measurable change in the chamber atmosphere. Both chambers are fitted with transparent Kel-F view ports. Details of this equipment have been previously reported (ref. 1).

In all exposure procedures, the composition of the atmosphere was verified by downstream analysis with a Beckman IR-5A infrared spectrophotometer, utilizing a 10 cm gas cell constructed of Teflon, with silver chloride windows. To test the integrity of gas concentrations too low for analysis, dummy exposures at high concentrations were set up in which experimental animals were simulated by a rolled wet towel.

Lethality Determination

After preliminary range finding tests, two atmospheric concentrations of each agent were selected and the lethal effect was examined in terms of the duration of exposure and the period of survival prior to death.

Lethality is expressed in terms of the minimum duration of exposure which is almost certainly lethal and the maximum exposure almost certainly permitting survival at each concentration. Since the slope of dose response relationship is so steep for most of the agents studied, precise determination of median or 95% lethal concentrations

would require immense numbers of animals with no real increase in useful information. Lethality of agents administered intraperitoneally has been estimated for comparison with the inhalation lethality of the compound. In most cases, it has been necessary only to establish whether the lethal intraperitoneal dose is in excess of some calculated amount, which requires very few animals.

Clinical Chemistry

Because the clinical assays used were of a survey nature, it was more suitable to have them conducted by a reliable commercial laboratory routinely engaged in such activities, rather than setting up the several procedures in our laboratories. The United Medical Laboratories of Portland, Oregon carried out the analyses, using the basic method for each determination identified in the following list:

Serum Protein Fractionation -- Grunbaum, B. W., J. Zec and E. L. Durrum, "Application of an Improved Microelectrophoresis Technique and Immunoelectrophoresis of the Serum Proteins on Cellulose Acetate," Microchem. J., Vol. 7, pp. 41-53 (1963).

Urea Nitrogen -- Automated Procedure Performed on a Technicon Autoanalyzer, Skeggs, L. T., J. Clin. Path., Vol. 28, p. 311 (1957). Marsh, W. H., B. Fingerhut and E. Kirsch, Amer. J. of Clin. Path., Vol. 28, p. 681 (1957).

Phosphorus -- Technicon Autoanalyzer, Automated. Fiske, C. H. and Y. Subbarow, J. Biol. Chem., Vol. 66, p. 375 (1925). Modified Procedure.

Sodium and Potassium -- Automated procedure using Technicon Autoanalyzer and Technicon flow-through flame photometer. Isreeli, J., M. Pelavin and G. Kessler, Technicon Research Laboratories, Chauncy, New York, N. Y. Acad. Sci., Vol. 87, Art. 2, pp. 636-649, (July 22, 1960).

Glucose -- Dubowski, K. M., "An o-Toluidine Method for Body-Fluid Glucose Determination," Clinical Chem., Vol. 8, p. 215 (1962).

Creatinine -- Technicon Autoanalyzer, Automated. Chasson, A. L., H. J. Grady, and A. M. Stanley, Amer. J. of Clin. Path., Vol. 34, No. I, pp. 83-88 (January 1961).

Calcium -- Bett, I. M., G. P. Frazer, "A Rapid Micro-Method for Determining Serum Calcium," Clin. Chim. Acta, Vol. 4, pp. 346-356 (1959).

Magnesium -- Perkin-Elmer Atomic Absorption Spectrophotometer, Model 303 is used for Mg analysis. Willis, J. B., Spectrochim. Acta, Vol. 16, p. 273 (1960).
Dawson, J. B. and F. W. Heaton, Biochem. J., Vol. 80, p. 99 (1961).

Chloride-- Technicon Autoanalyzer, Automated. Zall, D. M., Fisher, D., and M. O. Garner, Anal. Chem. Vol. 28, p. 1665 (1956).

Subjects were lightly anesthetized with ether at the time of sampling and 4 or 5 milliliters of cardiac blood were drawn. Appropriate anticoagulant, when required for an assay, was provided by the laboratory as a dry powder in the shipping tubes. Samples were transported to the laboratory by mail. It has been established by the laboratory that the conditions of transit do not affect the measurements of the parameters we have selected. The relative validity of determinations was checked at intervals by submission of duplicate samples under different identities. Absolute checks of some determinations have been made by addition of glucose, creatinine, urea or specific electrolytes to one portion of split samples. A modest effect of ether anesthesia has been found in some parameters, notably blood glucose, and is controlled by comparison with anesthetized, unintoxicated animals.

Methemoglobin and Hemoglobin Determination

In addition to the above clinical determinations, methemoglobin levels were measured following exposure of rats to the various gases.

Methemoglobin determinations were made by the method of Martin, et al., (ref. 3). Hemoglobin was measured by the Hycel procedure (ref. 4). A Beckman DB spectrophotometer with associated strip chart recorder was employed in these determinations.

Preparation of Erythrocytes for Dialysis of Fluoride

Erythrocytes were washed three times in 1.0% NaCl and suspended in sufficient 1.0% NaCl to replace the volume of plasma originally removed. Hemoglobin, methemoglobin and fluoride content of this suspension were measured. The cells were then removed and hemolyzed by addition of small volumes of glass distilled water. The

resulting suspension was centrifuged one hour at 20,000 x G, and the supernate brought to 12.5% saturation with ammonium sulfate at room temperature and allowed to stand for 20 minutes. After centrifugation again at 20,000 x G for one hour, the solution was brought to 52% saturation with ammonium sulfate, allowed to stand for 20 minutes, and re-centrifuged. The precipitated hemoglobin from the suspending medium was suspended in a small volume of glass-distilled water and dialyzed against 5 changes of distilled water over a period of 94 hours.

The hemoglobin precipitate was prepared for fluoride analysis by the method used for tissue analyses. The dialysis solutions were evaporated under mildly basic conditions for later determinations of fluoride.

Experimental Animals

Male Sprague-Dawley rats were obtained from the Pacord Research Laboratories, Beaverton, Oregon and From Berkeley-Pacific Laboratories, Berkeley, California. Animals were delivered at weights of approximately 200 grams and were maintained on a standard laboratory diet until the desired weight was attained.

Dogs were obtained from a county animal pound by legally established procedures.

Sheep blood was obtained from animals in the experimental flock of the Department of Veterinary Medicine, Oregon State University.

SECTION III

TOXICOLOGY OF SELECTED FLUORIDE COMPOUNDS

TOXICOLOGY OF NITROGEN TRIFLUORIDE (NF_3)

INTRODUCTION

Perhaps the most remarkable characteristic of nitrogen trifluoride (NF_3) is its relatively low chemical reactivity, particularly when considered in company with such agents as the interhalogens. To react NF_3 with various aqueous systems required conditions which are presumably far more rigorous than can be found biologically. Hurst and Khayat (ref. 5) were able to degrade NF_3 with 4 N HCl to the extent of 64% and with 10.5 N HI to the extent of 72% in 160 hours at temperatures above 100° C. A variety of acids, bases, and some salts were found to react with NF_3 to a much lesser degree under these conditions. The solubility and reactivity of NF_3 in water is very limited (ref. 5), a factor which gives rise to immediate concern about the nature of distribution to be expected when NF_3 is introduced into a biological system. NF_3 can be induced to react with water vapor by sparking, to produce HF, NO and NO_2.

Our experience with NF_3 has confirmed that it is relatively inert in most situations. Early experience in handling this compound indicates, however, that contact of NF_3 with glass must be avoided, since the absolutely anhydrous conditions under which NF_3 will be nonreactive with glass could not be maintained in our exposure system. Contact with Kel-F oil and with organic valve components also was found to be hazardous in certain conditions. Diaphragm pressure regulating devices and Bourdon type gauges were damaged immediately upon contact with NF_3 at high pressure, apparently due to formation of nonelastic surface layers on the metals. These problems have been previously discussed at some length (ref. 1, 2).

The first report on NF_3 toxicology was included in a paper by Ruff in 1931, who indicated that while capable of inducing methemoglobinemia, the compound was limited in its toxicity (ref. 6). More recently, Torkelson, et al., examined the lethality of NF_3 to rats and found that at 10,000 ppm, 100% lethality could be expected by the end of a one-hour exposure. Methemoglobin was identified, but not measured. The LD_{50} resulting from intraperitoneal administration of NF_3 to rats in these studies was approximately 8-10 ml/kg (ref. 7).

Since NF_3 is inert in most situations, the mechanism by which it is made to react with hemoglobin has become of great interest in view of the extensive production of methemoglobin after inhalation or injection of NF_3. The oxidation of hemoglobin to methemoglobin requires the removal of 1 electron per heme sub-unit and NF_3 is in some way induced to accept or mediate the electron transfer process.

Our approaches to the resolution of this mechanism include efforts to determine the stoichiometry of the reaction of NF_3 with hemoglobin, the site on the molecule where the oxidation takes place, and the ease with which the reaction takes place as compared with NF_3 oxidation of non-biological reducing systems.

Several aspects of the behavior of hemoglobin in the intact animal exposed to NF_3 have been studied. Measurements have been made in intact animals of the rate at which methemoglobin increases under the influence of inhaled and intraperitoneally administered NF_3, and the rate of reduction of methemoglobin to functional hemoglobin following intoxication. In larger laboratory animals, the rate of absorption of NF_3 has been directly observed while following the time-course of methemoglobin formation, making it possible to estimate the molar relationships between NF_3 and methemoglobin in the intact animals. These experiments have also provided some insight into the effect of NF_3 partial pressure upon toxicity and of the ability of reductase systems to counteract low-level methemoglobin formation.

Considered in the context of general toxicologic investigation, the method developed for this determination established the actual amount of an inhaled toxic gas absorbed by a subject, a measurement which is rarely available in inhalation toxicology.

The molar relationship between NF_3 disappearance and methemoglobin formation has also been observed *in vitro*. The evidence obtained *in vitro* and *in vivo* indicate that 1 mole of NF_3 will oxidize more than 1 and possibly as many as 3 moles of heme, and that the site of oxidation is probably at the heme iron. The reaction proceeds with surprising ease considering the general inertia of NF_3 to most reducing agents in the absence of energy inputs such as sparking or radiation.

METHODS

Reaction of NF_3 with Hemoglobin In Vitro

The reaction of NF_3 with hemoglobin *in vitro* has been studied in a closed system in which the amount of NF_3 utilized and methemoglobin formed during each reaction (Figure 1) enables comparison. The volume of the system is about 30 ml, in which a concentration of NF_3 high enough to expedite the reaction with hemoglobin can be achieved with a quantity of the gas small enough that any disappearance will cause substantial change. The entire device is constructed of polyethylene except where Tygon tubing passes through the Sigma peristaltic pump.

The infrared cell through which the gas mixture is cycled is very simple; the two silver chloride windows are separated by and tightly compressed against a 1-5/8" Viton "O" ring so that the effective optical path is less than 1 mm. The distal window is drilled on either side of the light beam to accept polyethylene male Luer fittings for connection with the tubing of the reaction chamber.

In a typical experiment, erythrocytes are washed in a Krebs-Ringer phosphate buffer at pH 7.4 and then osmotically ruptured. An effort is made in this process to maintain the highest possible content of red blood cells. The hemolyzed cells are centrifuged to remove gross cellular debris and the crude hemoglobin solution is stored under refrigeration. Whole blood or intact cells in buffer may also be studied in this manner, but in order to develop information on the stoichiometry of the reaction of NF_3 with hemoglobin, it is necessary to minimize or eliminate the activity of the methemoglobin reductase system by excluding glucose from the system.

Proper assembly of the unit prior to each experiment is assured by operation with dilute NF_3 or sulphur hexafluoride (SF_6) to detect leakage. No loss of NF_3 due to solubilization in water or the materials of construction has been detected.

When the integrity of the system has been established, it is thoroughly flushed with the diluent gas of choice and 5 or 6 milliliters of hemoglobin solution of known concentration are placed in the reaction vessel, along with a small Teflon covered magnetic stirring bar. The total amount of hemoglobin represents 0.1 to 0.2 millimoles of heme. One to 5 milliliters of NF_3 are transferred as a gas in an ordinary disposable plastic syringe, taking care to prevent diffusion of NF_3 out

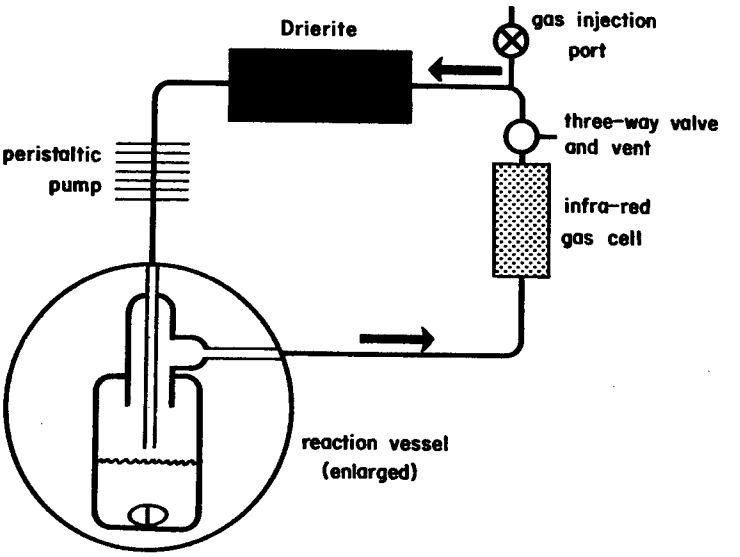

Figure 1. Apparatus for Measuring Uptake of NF_3 by Erythrocytes and Hemoglobin Preparations

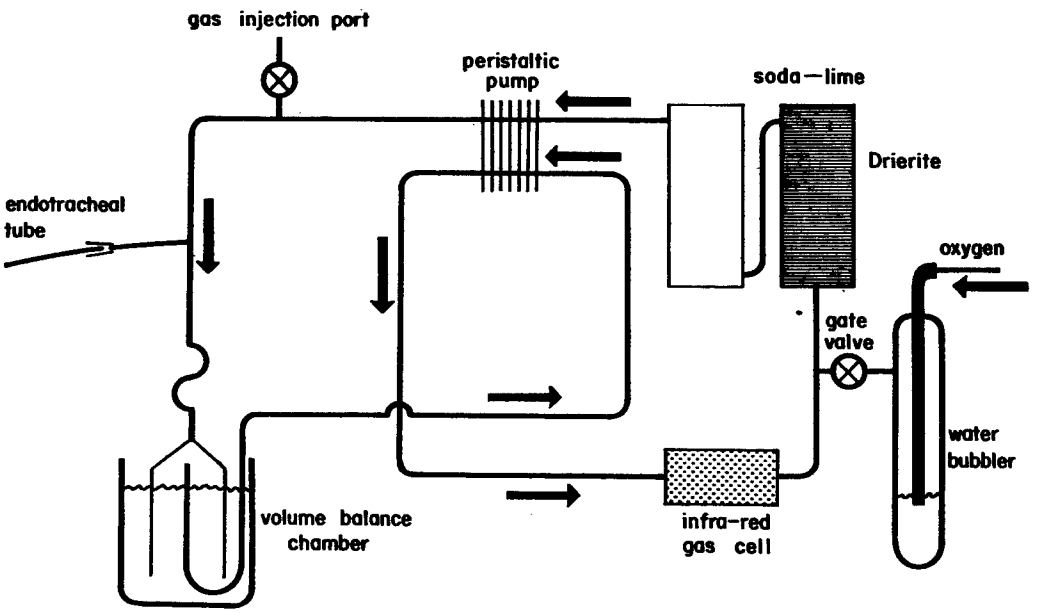

Figure 2. Apparatus for Measurement of NF_3 Uptake by Intact Large Laboratory Animals

of the syringe during the moment of transport from the storage source to the injection valve. NF_3 is injected slowly, displacing atmosphere through the entire system and finally through the infrared cell to assure that no NF_3 is lost in the process. The system is then closed and the pump started.

The optical density at 11.02 μ measured by the infrared spectrophotometer at the beginning of the reaction represents 100% of the amount of NF_3 introduced. This level is related to the volume of gas injected, as established by previous calibration of syringes and correction to standard conditions. The reaction of NF_3 with the polyethylene and neoprene of which the syringe is constructed has been determined to be insignificant over a period of at least two weeks.

During the course of the reactions, which usually consume 1-4 hours, absorption at 11.02 μ is continuously recorded on a strip-chart recorder. Spectra are periodically traced in order to establish that the integrity of NF_3 remaining in the system has not changed. During some experiments, small amounts of SF_6 were added as a part of the diluent atmosphere to assure that leaks did not develop during the exposure.

The reactions of NF_3 with solutions of cysteine and ferrous sulfate were also studied in this apparatus. These compounds, as representative organic and inorganic reducing agents, were used to provide some index of the spontaneous reactivity of NF_3. Experiments were conducted by the same procedures under which reaction with hemoglobin was followed. pH was adjusted to 7.0 in each case.

Uptake of NF_3 by Intact Animals

A device similar in principle to that just described is linked to an anesthetized dog by an endotracheal tube with an inflatable cuff (Figure 2). This closed system includes a chamber for compensation of respiratory volume changes, columns for removal of water and carbon dioxide from the recycled atmosphere, and an oxygen valve sensitive to pressure changes of 1 mm water to automatically maintain the oxygen partial pressure. The cycled gases pass through an infrared gas cell of 10 cm path length which allows continuous observation of the change in the NF_3 atmosphere.

When the animal has been coupled to the system, a few milliliters of sulfur hexafluoride are introduced. This gaseous compound is non-toxic and essentially insoluble in aqueous systems (ref. 8), and

has a sharply defined peak of absorption at 10.6 μ. If the concentration of SF_6 does not change during the experiment, it may be assumed that no leaks occurred. The seal between the trachea and the catheter is particularly vulnerable to bypassing of gases.

Blood samples are periodically obtained from the animal for methemoglobin determination during the period of NF_3 utilization. Each sample is obtained by venipuncture with a 26 gauge needle which causes very little vascular trauma over the relatively short periods of these experiments. For long-term experiments, a silicone rubber cannula may be inserted into any available peripheral vein for the duration of the work.

Data on the rate of formation of methemoglobin in the intact animal is corrected by establishing the rate of reduction after methemoglobin formation has been stopped. It is assumed that the true rate of formation represents the observed apparent rate, plus the rate of methemoglobin reduction, which is relatively linear. (Figure 3).

Blood volume determination is prerequisite to determination of the total amount of hemoglobin available for reaction with NF_3 in the intact animal. This measurement was made with I^{125} labeled radioiodinated serum albumin according to standard procedure (ref. 9).

Exposure of Whole Blood and Cells to Dynamic Atmospheres of NF_3

The behavior of blood or suspensions of erythrocytes in dynamic atmospheres of NF_3 was studied in the small exposure chamber described on page 4. A porcelain evaporating dish was placed in the chamber with a Teflon covered stirring bar and a stirring motor was placed just beneath the chamber. A small Teflon tube inserted from the outside into the dish enabled introduction of the blood and withdrawal of samples.

Observation of the Time Course of Methemoglobin Formation and Reduction

Blood samples for methemoglobin determination during the recovery period after NF_3 inhalation, and through the entire cycle of methemoglobin formation and recovery after intraperitoneal NF_3 were obtained by repeated heart puncture of individual rats. The volume obtained was approximately 0.3 ml at each sample. Sample intervals were 20-30 minutes. Such repeated sampling appeared to have no gross ill effects.

Uptake of Fluoride Ion by Methemoglobinemic Erythrocytes In Vivo

Four rats were administered 40 mg sodium nitrite intraperitoneally

to induce methemoglobin formation. At 20, 35 and 50 minutes after sodium nitrite administration, repeated doses of 10 mg of sodium fluoride were administered intraperitoneally, to each rat. The animals were anesthetized and exsanguinated at 60 minutes after the beginning of the experiment. Blood cells were separated, washed and assayed for fluoride according to procedures identified in the section on general methods.

RESULTS AND DISCUSSION

Symptoms and Lethality

Exposure to 1% NF_3 (10,000 ppm) usually required 60-70 minutes for lethal effect. In every case where measurement was made, the methemoglobin concentrations at the time of death were 60-70% of the available hemoglobin. The exposures described in Table I were made at several different times over a period of months which may account for the variation in time required for lethal effect among the groups of animals used. In most cases, the time between first observed death or major distress and the time nearly all or all animals succumbed was about 10 minutes.

TABLE I

Lethality to Rats* of Inhaled NF_3, 10,000 ppm in Air

Exposure	Duration	Lethality dead/total	Duration	Lethality dead/total
1	55 min	1/5	65	3/5
2	50	0/5	60	4/5
3	45	1/5	50	4/5
4	55	0/5	65	5/5
5	60	1/5	75	5/5
6	45	2/5	55	5/5
7	60	2/5	75	4/5
Average	53	1/5	63.5	4.3/5 (86%)

*Rat weights were 320-360 grams.

We have arbitrarily defined an acute exposure as less than one hour in duration. Since a 1% concentration of NF_3 can be considered as massive contamination even though requiring an hour to cause lethality, the alternate concentration chosen was at a lower level, 0.4%, which required a period longer than one hour. There was no lethality from exposures to 0.4% for 3 hours or less, and the ten animals exposed to this concentration all died within 3.75 hours.

Comparison of exposures to 1% and 0.4% NF_3 in air show that as the concentration was lowered, the product of concentration x duration of exposure became much greater. (At 0.25% NF_3 in air, dogs were able to reduce methemoglobin faster than it could be formed and presumably survive until chronic effects appeared.) Exposures considerably below the lethal level caused no apparent distress to rats other than accelerated respiration, probably due to direct irritation by the agent. Animals exposed to nearly lethal amounts of NF_3, on the other hand, showed very definite respiratory distress as well as generalized depression to the extent that they were non-responsive to external stimulation. The methemoglobin formed was superficially detectable in eye color and in the coloring of the mucous membranes and extremities. When very severely affected, the animals often collapsed, lying in unnatural positions and occasionally were incoordinate or convulsive. All severely affected animals urinated heavily, and almost uniformly demonstrated pronounced photophobia. Whether this was related to local irritation or failing oxygen supply is not known. It is possible that the latter symptom may serve as a crude index of the level of intoxication in a clinical situation.

To some extent, short-term blood pressure changes in the albino rat may be estimated on the basis of the intensity of eye color, which depends entirely upon the integrity of the superficial circulation. On this basis it would appear that severely affected animals suffer rather drastic fluctuations in blood pressure. In an animal showing a massive methemoglobinemia with very dark eye color, the eye may momentarily become very pale, presenting much the same appearance as a dead rat for a short period of time. Nonetheless, the subject animal rarely fails to recover if it survives the exposure more than 1-2 minutes. By 20-25 minutes after approaching a state of collapse caused by barely sublethal exposures to NF_3, animals were usually exploring the cage and dressing their fur.

The LD_{50} of NF_3 gas injected intraperitoneally is about 8.25 ml (0.37 mmole)/kg (Table II). Methemoglobin concentration reaches a maximum within 30 minutes after injection, and subsequently reduction to hemoglobin is nearly complete about 80 minutes after injection.

TABLE II

Lethality to Rats of Undiluted NF_3 Administered Intraperitoneally

Dose ml/kg	Number dead/ Number Injected
7.2	0/4
8.6	3/4
10.4	4/4

LD_{50} 8.2 (7.5-9.0) 95% (ref. 10)

A paradox appears in comparing the effects of inhaled and intraperitoneally administered NF_3. Death caused by lethal intraperitoneal doses of NF_3 occurred 2-3 hours after methemoglobinemia was dispelled. Animals subjected to NF_3 inhalation, on the other hand, almost always survived if alive at the end of the exposure, and animals dying of NF_3 inhalation uniformly had very high, presumably intrinsically lethal levels of methemoglobin. It seems possible, with these circumstances, that other pharmacological effects of NF_3 may exist.

Reactions of NF_3 with Hemoglobin In Vivo

Three in vivo experiments have been completed with anesthetized dogs which provide estimates of the stoichiometry of NF_3 reactions with hemoglobin in the intact animal. These experiments incidently demonstrate a method of measuring the actual uptake by experimental animals of gaseous toxic materials.

The data indicate a molar ratio of methemoglobin formed to NF_3 removed from the atmosphere of about 3 or 4 to 1. These values are slightly higher than those we have observed in in vitro experiments, but demonstrate without doubt that a mole of NF_3 will oxidize or mediate the oxidation of several moles of heme. It should be noted that almost all sources of experimental error in these experiments involve potential loss of NF_3 or its uptake by other systems. Measurement of methemoglobin, on the other hand, is subject to limited deviation either up or down, from standard determinations. This means that the ratios observed can only be increased by experimental defects.

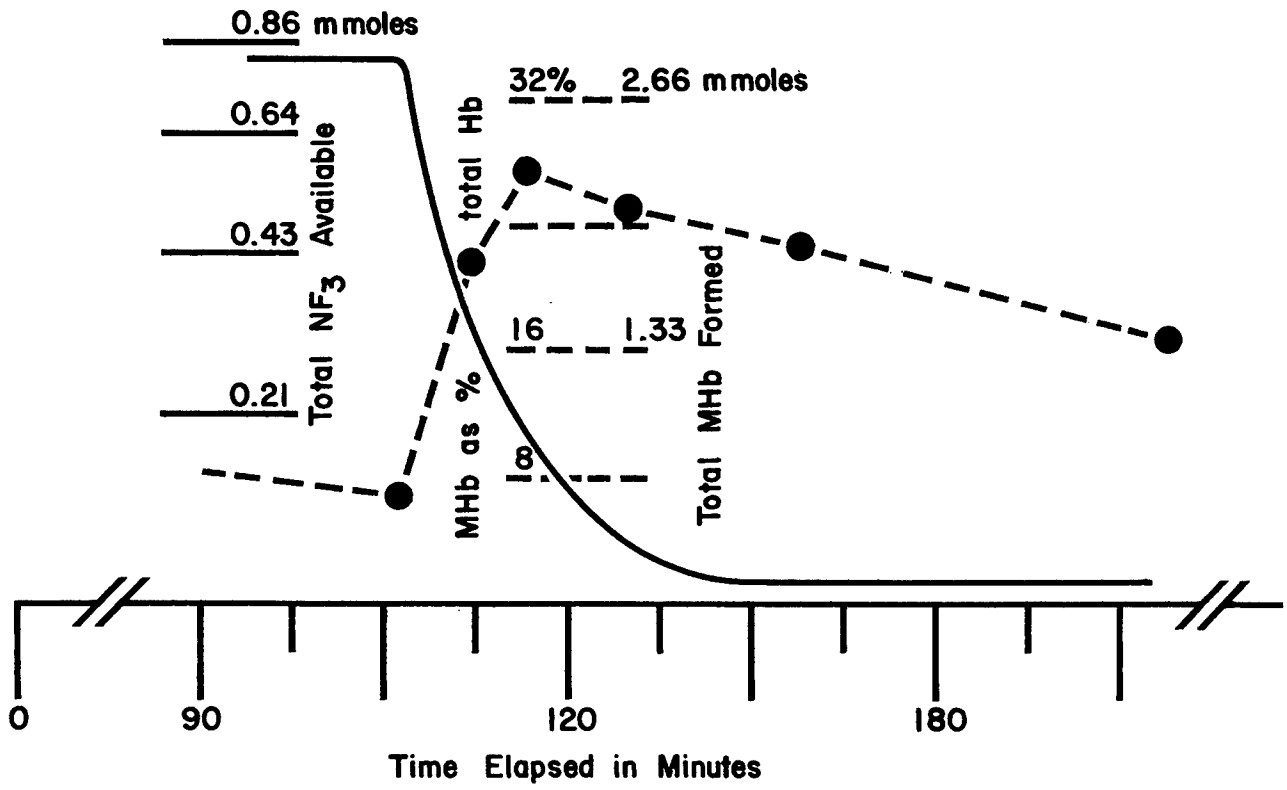

Figure 3. Uptake of NF_3 (—) Associated with Formation of Methemoglobin (●--●) During Intoxication by Inhalation of NF_3 Gas.

One such experiment is described graphically, to illustrate the rate at which the interaction between NF_3 and hemoglobin takes place (Figure 3). The initial methemoglobin level is at about 7% because the subject was recovering from an experiment begun 90 minutes previously which had failed due to leakage of NF_3. During the initial phase of the experiment, lasting 23 minutes, 0.46 millimoles of NF_3 were taken up by the animal from a total of 0.82 millimoles present in the experimental atmosphere. The increase in methemoglobin was equivalent to 1.93 millimoles of oxidized heme after correction for reductase activity, representing a ratio of 4.2 moles oxidized heme/mole NF_3 removed from the atmosphere. Recovery from methemoglobinemia began while NF_3 uptake was still in progress and the uptake of NF_3 ceased while measurable amounts of the gas still remained in the system. In two other experiments, ratios of 3.0 and 3.5 moles of heme oxidized/mole NF_3 were determined. Several other experiments which were considered incomplete for various reasons produced similar results.

The maximum rate of methemoglobin formation under these circumstances is on the order of 1%/minute of total hemoglobin, indicating that in a sustained atmosphere, a lethal methemoglobin level would be reached in about 1 hour, as is the case with rats. Evidence in these experiments indicated that the formation of methemoglobin is a linear function with respect to time at NF_3 concentrations of about 1%.

It has not been possible to answer the question of the relationship between the insolubility of NF_3 and transport in the extracellular circulation. A single brief observation has shed some light on this question, however. A dog coupled by an endotracheal tube to the closed system devised for the experiments just discussed was administered 2.4 ml of gaseous NF_3 intravenously over a 90 second period. During the seven minutes after the injection was begun, 1.1 ml of this gas emerged from the lung. The fate of the remainder was not determined, since no comparable control administration of a non-reactive gas such as SF_6 which might indicate trapping in emboli could be made at the time. A detectable slight increase in methemoglobin occurred, indicating that at least a limited amount of the gas was brought into contact with red blood cells. The principal value of the experiment was in confirming that little or no capability exists for NF_3 transport as a soluble gas. Further speculation is of no value at present.

Reactions of NF_3 with Hemoglobin Solutions and Other Reducing Agents In Vitro

Several experiments were conducted in which NF_3, at concentrations up to 8.5%, was shown to react in a closed system with

a crude cell-free preparation of hemoglobin to form methemoglobin. Measurements of the loss of NF_3 from the atmosphere in the presence of these hemoglobin solutions show that the molar ratio of heme oxidized/NF_3 removed is between 2 and 3, somewhat less than observed in vivo (Table III).

When NF_3 was maintained in contact 0.18 M ferrous sulfate or 0.08 M cysteine for several hours, none of the gas was removed from the atmosphere. Apparently, substantial activation is necessary to these reactions, even though NF_3 reacts readily with hemoglobin.

TABLE III

Conversion of Hemoglobin to Methemoglobin by NF_3 in Air: In Vitro
Ratio of Methemoglobin Formed to NF_3 Utilized

Initial HB, μmoles	100	50	100	60	60	60	50	60
Initial NF_3, μmoles	223	78	89	89	89	89	89	89
%NF_3 in Atmosphere	8.5	3.0	3.5	3.5	3.5	3.5	3.5	3.5
MHb/total Hb	100	63	43	75	86	90	96	93
μmoles MHb Formed	100	32	44	43	51	53	48	55
μmoles NF_3 Utilized	32	19	14	13	21	26	27	18
$\frac{\text{MHb Formed}}{NF_3 \text{ Utilized}}$	3.3	1.7	3.1	3.3	2.4	2.1	1.8	3.0

Effect of NF_3 Upon Methemoglobin Concentration in Whole Cell, In Vitro

When whole sheep blood is exposed, with gentle stirring, to a 1% NF_3 atmosphere, the rate of methemoglobin formation is much slower than that in the intact animal. The highest rate observed was about 12% of total hemoglobin per hour, the lowest was 6% per hour and the rate of formation of methemoglobin was linear throughout the entire period in each experiment. The rate of reduction of methemoglobin in such pre-

parations is about 1 or 2% of total hemoglobin per hour, which can readily be determined and applied as a correction for each experiment.

Reduction of Methemoglobin

Data obtained by measuring the rate of methemoglobin reduction in vivo and by whole blood in vitro illustrate differences among the effects of NF_3, N_2F_4 and sodium nitrite. The methemoglobin concentration of blood withdrawn from nitrite-treated animals and incubated in vitro was reduced from about 70% to about 10% in less than 4 hours, which is comparable to the in vitro rate of reduction of blood from NF_3 treated rats. In vivo reduction in sodium nitrite treated rats progressed at less than half the rate observed after NF_3 or N_2F_4 intoxication, in which methemoglobin concentrations in excess of 50% of total hemoglobin are reduced almost to normal in about 2 hours. The difference might be attributable to residual distribution of nitrite through the body or its persistence in the circulation, either of which would tend to sustain methemoglobin formation and counterbalance the reduction process. As a nearly insoluble gas, NF_3 should be expected to leave the cells and lungs immediately when exposure ends. The lowered partial pressure of NF_3 should result in cessation of methemoglobin formation. Experiments with dogs have shown that there is no apparent continuation of NF_3-induced methemoglobin formation after NF_3 is removed; the level begins to decrease immediately upon removal of NF_3. We have also established that plasma from lethally exposed rats has no effect upon red blood cells from untreated rats.

Observations of rats indicate that as methemoglobin concentrations approach lethal levels, the reductase activity is quite rapid. Accurate rate measurements have not been made, however. In the experiments thus far conducted with dogs, the methemoglobin concentrations were held between 25 and 40% of available hemoglobin, and in this range, the rate of reductase activity in terms of percentage total hemoglobin turned over appears linear.

Elevated methemoglobin concentrations in rats which have been exposed to NF_3 usually return to a persistent resting plateau level which is measurably higher than the normal resting concentration of approximately 1% of total hemoglobin (Table IV). In dogs, 3-5% methemoglobin concentrations persist for up to two weeks following exposure to sufficient NF_3 to cause methemoglobin concentrations in excess of 25%.

This low level methemoglobinemia may represent an inhibition of methemoglobin reductase activity. A similar effect is caused by NO_2 inhalation, which, at concentrations from 20 to 1,000 ppm, causes a

long-standing methemoglobin formation in rats, to the extent of about 10%. The possibility that NF_3 forms some NO_2 *in vivo* might be considered since the reaction has been demonstrated under more rigorous conditions (ref. 5), but the available evidence is hardly sufficient to invoke this degradation scheme in animals.

TABLE IV

Long Term Recovery From Methemoglobinemia After NF_3 Intoxication

Animal		Days After Exposure			
		0*	2	4	7
		% of hemoglobin as methemoglobin			
41	10,000 ppm	40.8	3.5	2.5	5.2
43	NF_3/55 min	48.0	4.7	5.6	1.8
44		47.4	3.4	3.5	2.4
45		46.2	4.4	4.1	3.0
Average		45.6	4.0	3.9	3.1
46	Control	1.0	0.8	0.8	1.4
47		1.0	0.8	0.4	1.0
48		0.6	3.6	0.8	2.2
49		0.8	0.4	0.1	1.0
Average		0.8	1.4	0.7	1.4

*Sampled immediately at end of exposure

It is unlikely that NF_3 could remain in the cell or in the circulation in sufficient concentration to cause such a continual formation of methemoglobin. We have established that it requires a concentration of NF_3 in the atmosphere greater than 0.25% to induce methemoglobinemia above basal levels in dogs, and that uptake of NF_3 ceases while its concentration is reduced to about 0.05% NF_3 in the atmosphere. At NF_3 concentrations below 0.25%, methemoglobin formation is slower than the countering reduction capability; NF_3 uptake will consequently continue while methemoglobin levels recede or stabilize.

Whatever the cause of the residual methemoglobin following NF_3 intoxication, interference with conversion of subsequently induced high levels of methemoglobin seems limited. Rats recovered from NF_3 intoxication appear to become no more susceptible to second exposure, and dogs appear to respond similarly in successive experiments.

Relationship Between Fluoride and Methemoglobin in Erythrocytes After Exposure to NF_3

Significant amounts of the fluoride in red blood cells of animals intoxicated in an atmosphere of 1% NF_3 for 55 minutes were found to remain apparently bound to methemoglobin, after extensive dialysis of purified preparations of the hemoglobin and methemoglobin from these cells (Table V). Erythrocytes from rats made methemoglobinemic with sodium nitrite and then treated with heavy non-lethal doses of sodium fluoride were found to be free of fluoride, however.

TABLE V

Relationship Between Methemoglobin and Bound (non-dialyzable) Fluoride of Blood from Rats Intoxicated with NF_3

	μ Moles Meth-emoglobin	μ Moles Oxidized Heme	μ Moles F^- Bound	Bound F^-/ Oxidized Heme
A-1	2.1	8.4	0.89	0.106
A-2	1.8	7.2	0.6	0.083
B-1	0.82	3.3	0.62	0.18
B-2	0.34	1.34	0.24	0.18

The data from these experiments provide a starting point for speculation on the site of attack of NF_3 upon hemoglobin. The ability of methemoglobin *in vitro* to bind fluoride (and other anions) is classic,

but the concentrations of fluoride required are very high when compared with the concentration permitted in vivo by the toxicity of sodium fluoride. In spite of the failure of fluoride to accumulate in erythrocytes containing methemoglobin after sodium fluoride administration, as much as 0.2 equivalent of fluoride remains in the cells of rats treated with NF_3 for each mole of oxidized heme (Table VI). How much more fluoride has been bound less securely is not known. On the basis of the limited number of observations made, the ratio appears reasonably constant at several concentrations of methemoglobin. Exceptions were only in the initial measurement at a low methemoglobin level, and in one sample in which reduction to hemoglobin was nearly completed. In the latter case, substantial fluoride remained in the cells after reduction of methemoglobin. This observation has been verified many times in studies of fluoride distribution following exposure to NF_3.

TABLE VI

Relation Between Erythrocyte Fluoride Content and Level of Methemoglobin In Washed Erythrocytes From Rats Exposed to 1% NF_3
(Each observation represents an independent experiment)

Exposure Duration (Minutes)	Time of Sample After Exposure (Minutes)	MHb as % Hemoglobin	μMole Cell F/100 ml Blood	μMole Oxidized Heme/100 ml Blood	F/Oxidized Heme
20	3	28	22.7	262	.08
35	1	60	71	556	.13
35	2	44	71	356	.2
50	9	64	76	487	.16
50	2	60	81	468	.17
50	80	33	43	281	.15
50	154	6.7	40.4	53	76.0

These facts indicate that either a substantial barrier to fluoride transport into the cell exists in the normal animal, or that the fluorine atom is liberated during the reduction of NF_3 very close to the required site of attachment to heme iron, and by virtue of such preference, is able to complex with methemoglobin. It also suggests that the electron transfer associated with iron oxidation was accomplished either at the same time or just before breakage of NF_3. Any appreciable delay of methemoglobin formation after release of fluoride should considerably lessen the chance that the iron-fluoride complexing would take place.

When red blood cells are incubated in vitro in Krebs-Ringer Phosphate buffer, pH 7.4, under a dynamic atmosphere of 5% NF_3, a fairly constant relationship between total cell fluoride and the amount of methemoglobin formed can also be demonstrated.

These high ratios of cellular fluoride to oxidized heme are curious. In conducting the dialysis experiments, it was clear that most cellular fluoride was lost in the washing and precipitation process since no fluoride was detectable in the dialysate. In addition, cells from intoxicated animals which had been washed only, contained methemoglobin and fluoride in a similar ratio to that found after dialysis (Tables V, VI). In these experiments, the ratios are an order of magnitude higher, and, in fact, exceed 1.0 (Table VII), suggesting a reaction with some factor other than the heme iron, at the high concentration of NF_3 employed in vitro.

TABLE VII

Ratio of Fluoride to Methemoglobin in Washed Erythrocytes Incubated Under a Dynamic Atmosphere of 5% NF_3

Duration of Exposure (Minutes)	% Hb as MHb	Grams % MHb/ 100 ml	μMoles Heme Oxidized/ 100 ml	μMoles Cell Fluoride/ 100 ml	Cell F^-/Oxidized Heme
0				2.6	
45	10.9	1.33	83	133	1.6
135	22.9	2.91	182	212	1.17
240	30.1	3.89	244	337	1.38
360	31.8	4.14	259	349	1.35

NF_3 Effects Upon Hemoglobin Concentration in Blood

Total hemoglobin concentration was raised for a period of several days by severe acute NF_3 intoxication (Table VIII). The response of animals within both control and intoxicated groups was erratic, possibly due to fluctuations of shorter periods than the sampling interval. Such variable data are not usually meaningful, but in this case the average total hemoglobin of treated animals was clearly increased by the fourth day, while that of control animals decreased. The reasons underlying these differences are not known. Higher initial control values might be attributed to splenic release of red cells during the first bleeding. Animals treated with NF_3 are usually depressed when brought out of the exposure chamber and would not be expected to respond with adrenergic activity as extensively as control animals. After this period, however, the response to the insult of bleeding must be considered equal in both groups.

TABLE VIII

Hemoglobin Concentration in Rats Following NF_3 Intoxication
(NF_3 10,000 ppm for 15 Minutes)

Days After Exposure	0*	2	4	7	Initial Hemoglobin gm/100 ml
	% Change from Initial Hb Concentration				
Exposed Animals					
41	0	9.5	16.8	10.8	17.8
43	0	- 6.6	7.8	- 5.8	16.6
44	0	10.2	26.8	6.3	16.7
45	0	5.0	21.8	1.2	17.5
Average		4.5	18.3	3.1	17.1
Control					
46	0	19.0	- 4.3	- 3.7	20.2
47	0	0.5	-23.2	-17.8	16.2
48	0	-22.1	- 3.5	10.5	20.0
49	0	- 8.0	-19.0	-12.0	17.2
50	0	11.3	-14.0	- 4.5	17.8
Average		9.0	-12.8	- 5.7	18.3

*Sampled immediately at end of exposure

It is doubtful that loss of the small amounts of blood withdrawn is of importance in this change, but trauma of sampling may account for the eventual decrease of hemoglobin evidenced in control animals and which may be masked in intoxication. It would appear at present that there would be value in studying further the influence of NF_3 and methemoglobinemia upon hematopoietic responses to determine first whether this effect is simply a function of methemoglobinemia and, if not, whether some pharmacologic effect of lower doses of NF_3 might be sought.

In experiments of shorter duration, the effects seen during the first 24 hours were relatively limited and were paralleled, if not associated, with a change in packed cell volume. At 6 hours after exposure, a decrease of about 15% in average hematocrit was accompanied by about an 8% decrease in hemoglobin. By the end of a 24 hour period, however, the hemoglobin content increased in treated animals to an average of about 13.5% more than the initial level.

Effects of NF_3 on Blood Chemistry

NF_3 exposures resulted in sharp increases in blood glucose (see Table IX), which disappeared shortly. These may have been associated with adrenal medullary discharge resulting from confinement in an irritant atmosphere. Of greater curiosity is the transient rise of nearly 60% in serum creatinine and about 90% in blood urea nitrogen (BUN), and a more persistent increase in the α-1-globulin fraction (Table IX). These changes are usually thought to reflect renal malfunction; but only the plasma protein alteration persisted through the observation period, whereas physical damage to the kidney should cause long-term changes in all such parameters. It is possible that a pharmacologic effect by NF_3 upon filtration or secretion mechanisms may exist, but this question has not been probed.

TABLE IX. Effects of Inorganic Fluoride Oxidizing Agents Upon Selected Blood Constituents Expressed as: average value (range) number of animals

		Days Post Exposure			
Agent	Parameter	0	2	6	20
NF_3 1%/30 min	BUN mg/100 ml	20(18-24)6	25(20-36)4	41(37-48)5	22(17-27)6
Control	"	20(16-25)13			
NF_3 1%/30 min	Serum Creatinine mg/100 ml	1.6(1.4-1.7)6	1.2(1.6-1.1)4	1.1(0.9-1.4)4	0.9(0.7-1.2)7
N_2F_4 1%/15 min	Serum Creatinine	1.3(0.9-1.6)4	1.5(1.3-1.9)4	1.1(1.0-1.2)3	0.9(0.9)4
Control	"	0.7(0.6-0.9)7			
NF_3 1%/30 min	Blood Glucose mg/100 ml	339(296-386)6	181(115-282)4	124(113-141)5	105(960-123)7
N_2F_4 1%/15 min	"	132(76-196)4	160(113-230)4	117(94-143)4	119(86-184)4
ClF_3 0.04/30 min	"	320(266-406)6	182(114-286)6	144(123-187)3	116(81-132)7
BrF_5 0.05/20 min	"	165(104-258)6	153(110-238)5	145(123-169)5	110(97-122)5
OF_2 15 ppm/10 min	"	181(159-233)6	148(127-173)6	154(142-183)4	142(117-196)5
Control	"	113(92-127)10			
NF_3 1%/30 min	α-1-globulin gm/100 ml	0.48(0.41-0.60)5	0.54(0.36-0.78)4	0.77(0.74-0.82)3	0.61(0.50-0.68)3
BrF_5 0.05%/20 min	"	0.60(-.38-0.94)5	0.44(0.25-0.87)5	0.34(0.26-0.40)4	0.33(0.24-43)5
OF_2 15 ppm/10 min	"	0.60(0.46-0.80)5	0.56(0.37-0.70)5	0.51(0.37-0.68)4	0.96(0.68-1.77)4
Control		0.37(0.16-0.60)7			

CONCLUSIONS

It cannot be stated with certainty that the sole basis of the acute effect of inhaled NF_3 upon mammals is formation of methemoglobin and consequent failure of oxygen transport, but the indirect evidence available strongly favors such a case. Each animal observed at the terminal stages of NF_3 intoxication has been found suffering from 60-70% conversion of hemoglobin to methemoglobin which is presumably a lethal level. When no appreciable damage exists other than high methemoglobin levels, a living intoxicated animal should be expected to recover and survive indefinitely if reduction of methemoglobin begins at once and if his oxygen requirements do not increase. This is true of animals exposed to NF_3. With the exception of animals that are moribund and die a few moments after exposure ends, any animal surviving the exposure survives indefinitely.

At the same time, there is a reasonable suggestion that other toxic effects may be caused by NF_3. Lethal intraperitoneal doses of NF_3 do not cause death until after the induced methemoglobinemia has been dissipated. There is also non-specific evidence of temporary renal impairment during and shortly after exposure.

It is evident that each mole of NF_3 oxidized more than one mole of heme, but the mechanism and sequence of this reaction is unclear. The limited evidence available suggests that NF_3 is reduced at the site of the heme iron, but the source of energy necessary to initiate this reduction is unknown. Further definition of the mechanism by which NF_3 interacts with hemoglobin should provide important insight into the reactive behavior of hemoglobin.

Of considerable interest as well is the process by which the nearly insoluble NF_3 reaches the erythrocyte in order to react. Some speculation on this transport problem is possible when examining the character of the compound and its observed behavior. We suggest that molecular NF_3 is diffused directly into erythrocytes as the cells traverse the pulmonary capillary bed, and that little, if any, NF_3 is carried in solution to be diffused into cells during circulation. Erythrocytes passing through capillaries are tightly confined, and it may be expected that almost all extra-cellular water is physically stripped away from the cell surface, which will be directly apposed to the lining of the capillary. A thin, possibly monomolecular, layer of water should be expected to remain firmly attached to the wall of the cell and probably may be considered a functional component of the wall. If this is the case, the gas may be

expected to permeate the membrane regardless of the problem of water insolubility on the distal side. At this point, NF_3 may be considered an inert gas, to which the pulmonary blood gas barrier is quite permeable (ref. 11). Further, there should be an effective concentration gradient induced by hemoglobin functioning as a chemical receptacle for the oxidizing agent.

TOXICOLOGY OF TETRAFLUOROHYDRAZINE (N_2F_4)

INTRODUCTION

Early in our studies it was recognized that tetrafluorohydrazine (N_2F_4) was so unstable in the atmosphere that there was some doubt whether exposed animals would ever come into contact with the parent compound. Carson and Wilinski also recognized this problem and attempted to conduct their experiments in such a way that valid data could be collected (ref. 12). Studies of intoxication with N_2F_4 were therefore paralleled by, and became dependent upon, chemical studies begun in an effort to identify the terminal products to be expected of the reaction of N_2F_4 with moist air, and to learn the rate at which such products form.

The difficult problems of achieving nearly absolute exclusion of oxygen and water from the reaction vessel interfered consistently with progress of these studies. Both conditions were necessary to establish the influence of these normal atmospheric constituents alone in the gas phase. The experiments have resulted in substantial qualitative and quantitative evidence describing the dilute gas phase reactions of N_2F_4. It is clear that if water is present, N_2F_4 diluted in air decomposes to nitrosyl fluoride, which in turn forms HF, NO, and NO_2 in the presence of water. Experimental evidence indicates that NO and NO_2 may both contribute to the acute inhalation toxicity of N_2F_4 and that the lethal effect is accompanied by if not caused by high levels of methemoglobin, probably caused by NO.

METHODS

Observations of dilute N_2F_4 reactions in the gas phase were made by infrared spectroscopy using a Beckman IR-5A instrument. The gas cell for the infrared system was machined from Teflon to a length of 10 cm and fitted with silver chloride windows. Swagelok fittings for 1/4" tubing were fabricated of Teflon and located 1 cm from each end. In assembling the cell, a 1 5/8" neoprene "O" ring is used as a compression gasket between the compression ring and the cell window. Windows and the Teflon cell body are in direct contact, with a very light film of #90 Kel-F grease at the outer edge of the contact surface. The compression frame is then evenly tightened to the maximum extent possible and the seal is accomplished by cold flow of the Teflon cell

body and the elastic character of the crystalline silver chloride windows. Less rigorous assembly will permit significant volumes of oxygen to enter the cell.

The gas ports were fitted for these studies with Teflon 2-way Hamilton gas-tight valves, in turn connected with flushing and exit lines. Reagents were introduced through a 0.2 mm (ID) Teflon flexible needle with Luer hub, which entered the cell through a friction sleeve fitting. A three-way Hamilton Teflon valve at the outer terminus of this tube allowed entry of gases directly from reservoirs or from syringes. Each of these ports was established as gas tight. Where necessary, fittings and syringe tips were bathed in dry nitrogen during transport and coupling operations to prevent entrance of oxygen.

The sample bay of the instrument was tightly hooded against light to protect the silver chloride windows from discoloration. The entire instrument was continually flushed with dry nitrogen admitted through the standard fittings provided and vented through the beam ports and light shield.

Reactions of N_2F_4 and its products were observed at dilutions based on N_2F_4 concentrations of 0.5 - 2% in air or nitrogen. Other diluent gases were examined, including CO_2, and the system of reactions appeared to proceed satisfactorily in each.

The various reactions observed included those of N_2F_4 in presence of oxygen, water, oxygen and water, and NO_2; NOF in presence of water and of NO_2; NO_2 in presence of N_2F_4; and NO in presence of water. In each case, reagents were admitted in very small and in excessive quantities.

Oxygen and N_2F_4 for injection into the reaction cell were stored in and displaced from a column filled with Drierite. Each end of a small polyethylene drying tube was fitted with 3-way Hamilton Teflon valves, and the tube charged with 40-mesh non-indicating Drierite. The proximal 3-way valve was coupled to the reservoir of oxygen or N_2F_4 according to the experiment, and to a disposable polyethylene syringe. The distal valve permitted exit into the reagent entry port or into a waste line. In operation, the syringe and drying column were thoroughly flushed with the required gas, and allowed to stand with valves closed. After drying, gas was displaced into the gas cell by injection of the desired quantity of the same gas into the opposite end of the drying tube. We established that neither the polyethylene syringe with neoprene plunger nor the Drierite caused any change in the infrared spectrum of N_2F_4, even after several hours of standing.

Water as vapor in saturated nitrogen gas was added by syringe to reaction mixtures. Nitrogen dioxide was drawn directly from storage into a glass syringe and transferred through a three-way valve into the gas cell. NO_2 gas may be considered self-drying and no water removal is necessary.

Molar absorption curves for N_2F_4 and NO_2 were established empirically by injection of known volumes of the gases into the infrared cell with calibrated syringes. In each case, the maximum added volume was less than 1 ml, in a chamber volume of 75 ml. The injection was made with an exhaust port open, which was closed immediately upon pressure equilibration. No standardization of NOF was attempted since our source could not be totally freed of NO_2. Since the proportion of NO_2 was variable with conditions of transfer, a curve by difference could not be extracted. NO has an exceedingly low absorption coefficient and cannot be measured in our experiments; we find that 50% NO in nitrogen has an optical density of about 0.5, which is confirmed in the literature (ref. 13).

The extent and rates of reactions were measured by coupling the infrared spectrophotometer to a Beckman stripchart recorder. By frequently shifting manually among the various desired wavelengths, the timecourses of concentrations of N_2F_4, NOF and NO_2* were plotted during each single reaction sequence.

Potential reactions of N_2F_4 with oxygen were also studied by standard high vacuum procedures. N_2F_4 was transferred from the reservoir tank through new stainless steel fittings which had been thoroughly "soaked" and flushed with N_2F_4. Swagelok fittings were used to provide metal to glass union. A finger type reservoir bulb with stopcock and standard taper fitting which could be mated to the vacuum manifold was dried and thoroughly flushed with dry nitrogen. For filling, the finger of the flask was cooled in liquid nitrogen and N_2F_4 was slowly passed in until a sufficient amount had been frozen out. The flask was transported in liquid nitrogen and coupled to the vacuum manifold, where N_2F_4 manipulation was carried out in the routine manner.

*Wavelengths for each compound were: N_2F_4, 10.45 μ; NOF, 5.4 μ and NO_2, 6.51 μ.

RESULTS AND DISCUSSION

Symptoms and Lethality of Acute N_2F_4 Intoxication

Inhaled N_2F_4 at concentrations of 1% (10,000 ppm) in air is lethal after about 25 minutes of exposure (Table X). Thirty minute exposures to 1% N_2F_4 were found to be lethal before the end of exposure in every case. Exposures at this concentration for 25 minutes usually resulted in death before removal from the exposure chamber or within 10 minutes after removal. Twenty minutes in this atmosphere was non-lethal. Lethally affected animals occasionally died within the few minutes after emergence from the chamber, but if this period was survived, death rarely occurred.

A rather specific gasping effect appeared shortly after the exposures began, which often persisted without change throughout the period of exposure and for hours or days thereafter. This gasping activity was a short, sharp heaving respiratory movement suggesting a histamine effect. We have, however, been unable to produce any relief by treatment with anti-histamines or with epinephrine.

TABLE X

Lethality to Rats of 1% N_2F_4 In Air

Duration of Exposure (Minutes)	Number of Animals	Dead/ Exposed
30	4	4/4
25	10	8/10
20	10	0/10
15	10	0/10

Methemoglobin levels approaching 65% are encountered at the end of lethal exposures to N_2F_4, but the peripheral cyanosis observed does not appear identical to that accompanying NF_3 poisoning; methemoglobinemia may be accompanied by peripheral circulatory failure.

The LD_{50} of N_2F_4 administered intraperitoneally is between 10 and 12 ml/kg, which is less than the amount which would be expected to contact the lung in a lethal exposure. The peculiar respiratory activity caused by inhaled N_2F_4 does not appear, but the respiration does become labored. It is at this point that a problem arises in understanding the physiological mechanism by which N_2F_4 exerts its lethal effect. Lethal intraperitoneal doses of N_2F_4 do not cause more than about 10% conversion of hemoglobin to methemoglobin. It is interesting that lethal effects observed by Carson and Wilinski were accompanied by methemoglobin concentrations of less than 10% and that the very early work done in this program provided similar information.

Effect of N_2F_4 on Blood Chemistry

Slight, transient elevations of serum creatinine and blood glucose were the only discernible changes among the clinical chemistry observations made. Both effects were more pronounced at 2 hours than immediately after exposure, but the significance of this change is unclear.

Chemistry of N_2F_4 Decomposition

The gas phase of reactions of N_2F_4 with oxygen in the presence of small amounts of water, which we have observed by infrared spectrophotometry, may be summarized generally by stating that N_2F_4 forms NOF, which in turn reacts to form NO_2. Presumably, HF is also a terminal product but it has not been measured directly.

The relative time course of decomposition and formation of these compounds in the gas phase is illustrated in the idealized diagram based upon our data, which appears in Figure 4.

This sequence may be extended over an hour or more, if limited oxygen and water are available to the reaction. When both are plentiful, the formation of NO_2 may reach its maximum within 45 seconds after admission of water to the cell; N_2F_4 will disappear within 25-30 seconds under these circumstances. True measurements of the rates of these reactions are probably impossible, since the time required for gas diffusion may become limiting. When the reaction is permitted to proceed at a maximum rate, or when carried out with excess water and limited oxygen, NOF disappears so rapidly that only traces may be detected.

If the mechanism of decomposition of N_2F_4 in air is considered as a homogeneous gas phase process without the above empirical evidence, the reaction might be expected to follow a different course:

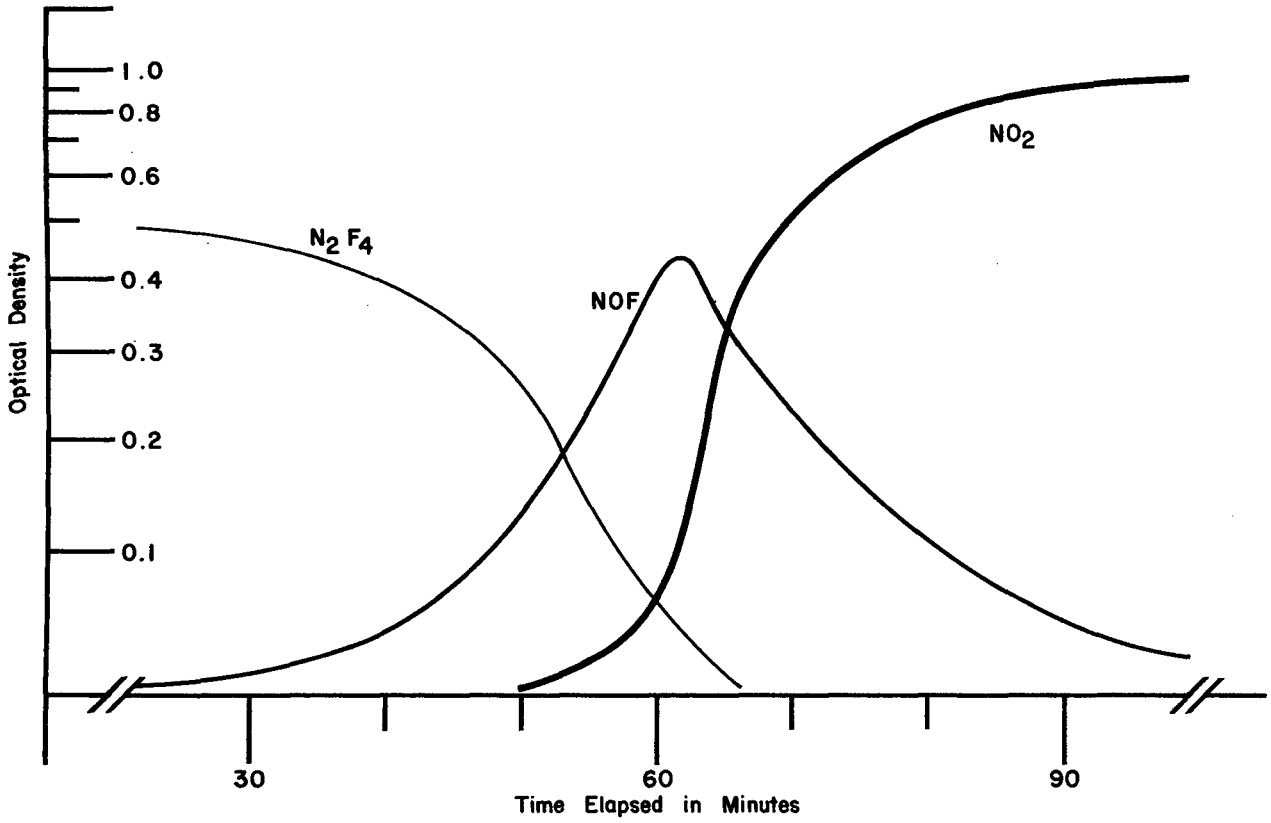

Figure 4. Typical Time Course of N_2F_4 Decomposition in Air with Low Moisture Content 23° C, 1 Atmosphere, Initial Concentration of N_2F_4 1.33%
Cell: Teflon with AgCl windows, 10 cm optical path, total volume 75 ml

$$2N_2F_4 + O_2 \rightarrow 2NOF + 2NF_3$$

The reaction as written is thermodynamically highly favorable, being exothermic overall to the extent of about 88 kilocalories per mole. Beach (ref. 14) reported observation of this reaction by infrared analysis in metal gas cells with sodium chloride windows. We have been able to show, however, that no reaction occurs between N_2F_4 and oxygen under anhydrous conditions in a Teflon gas cell with AgCl windows at ambient temperature and pressure. We could not detect NF_3 in the reaction mixture. Our attempts to examine the interaction of N_2F_4 and oxygen under high vacuum, in a Pyrex glass manifold system, and under anhydrous conditions have also failed totally, confirming the report by Sicre and Schumacher (ref. 15) who were unable to conduct this reaction in a water-free aluminum cell.

It is possible to suggest a mechanism on the basis of previous reports and our present observations which accounts for the behavior of N_2F_4 in a moist atmosphere. The equilibrium:

$$N_2F_4 \rightleftarrows 2NF_2 \cdot \qquad (1)$$

has been documented (ref. 16-20), and we may assume that $NF_2 \cdot$ is the reactive species. The initial reaction with oxygen is presumably:

$$\cdot NF_2 + O_2 \xrightarrow{(i)} NF_2O_2 \xrightarrow{(ii)} NOF + F + O \qquad (2)$$

This reaction is expected to be relatively slow. It is approximately 42 kilocalories endothermic; the rate-determining step may be the breaking of the oxygen-oxygen bond, which has an energy requirement of approximately 118 kilocalories/mole.

The monoatomic species formed in reaction (2) presumably would react further by dimerization or by one of the following:

$$\cdot NF_2 + F \xrightarrow{K_1} NF_3 \qquad \Delta H_r = -50 \text{ kcal}$$

or

$$\cdot NF_2 + O \xrightarrow{K_2} NOF + F \qquad \Delta H_r = -66 \text{ kcal}$$

Both reactions are highly exothermic, but since the experimental evidence shows that NF_3 does not form as an end product, and there is no reason to regard it as an intermediate, presumably a kinetic factor is responsible for this selectivity, i.e., $K_2 > K_1$.

If the products of reaction (2) are removed, the reaction must proceed to the right. It has been suggested (ref. 21) that NOF reacts vigorously with H_2O as follows:

$$2NOF + H_2O \rightarrow NO + NO_2 + 2HF \qquad (3)$$

This has also been demonstrated in the gas phase in our experiments. A stable, static dilution of NOF in dry air or nitrogen is abruptly converted to NO_2 when water is injected into the cell. When NOF, NO_2 and N_2F_4 are together in the gas cell, as during the intermediate phase of the slow decomposition of N_2F_4, introduction of oxygen saturated with water will cause an abrupt decrease and recovery of NOF concentration and a concomitant increase and decline in NO_2 concentration, which lags very slightly in time behind the change in NOF (Figure 5). The net change in concentration in each component relative to its overall rate of formation is zero.

Experimentally, little NO_2 is usually observed until the concentration of N_2F_4 has become very low, as illustrated in Figure 4. For explanation, suppose:

$$\cdot NF_2 + NO_2 \xrightarrow{(i)} NO_2NF_2 \xrightarrow{(ii)} 2NOF \qquad (4)$$

This reaction is reasonable, since NO_2 and $\cdot NF_2$ each contain a single unpaired electron. The possibility of the formation of NO_2NF_2 as an intermediate is further supported by work of Colburn's group which shows the formation of $NF_2 \cdot NO$ (ref. 22, 23). Thermodynamically, the overall reaction (4) is favored since it is exothermic to the extent of about 50 kilocalories/mole. Hurst and Khayat have presented evidence that the reaction between NO_2 and N_2F_4 is fairly rapid (ref. 5), and we have confirmed that this is the case.

When small amounts of NO_2 are added to dilute N_2F_4 in dry air or nitrogen, NO_2 disappears rapidly with a simultaneous decrease in N_2F_4 and an increase in NOF. If NO_2 is added until N_2F_4 disappears, NO_2 will remain, and no further increase in NOF occurs with increased addition of NO_2. Similarly, the addition of N_2F_4 to an NO_2 dilution results in disappearance of N_2F_4, a decrease in NO_2, and an increase in NOF.

The change in molar concentration of the respective gases fits the conditions of equation (4) reasonably well when NO_2 is added to N_2F_4. NO_2 as a product, however, is not easily measured. If the

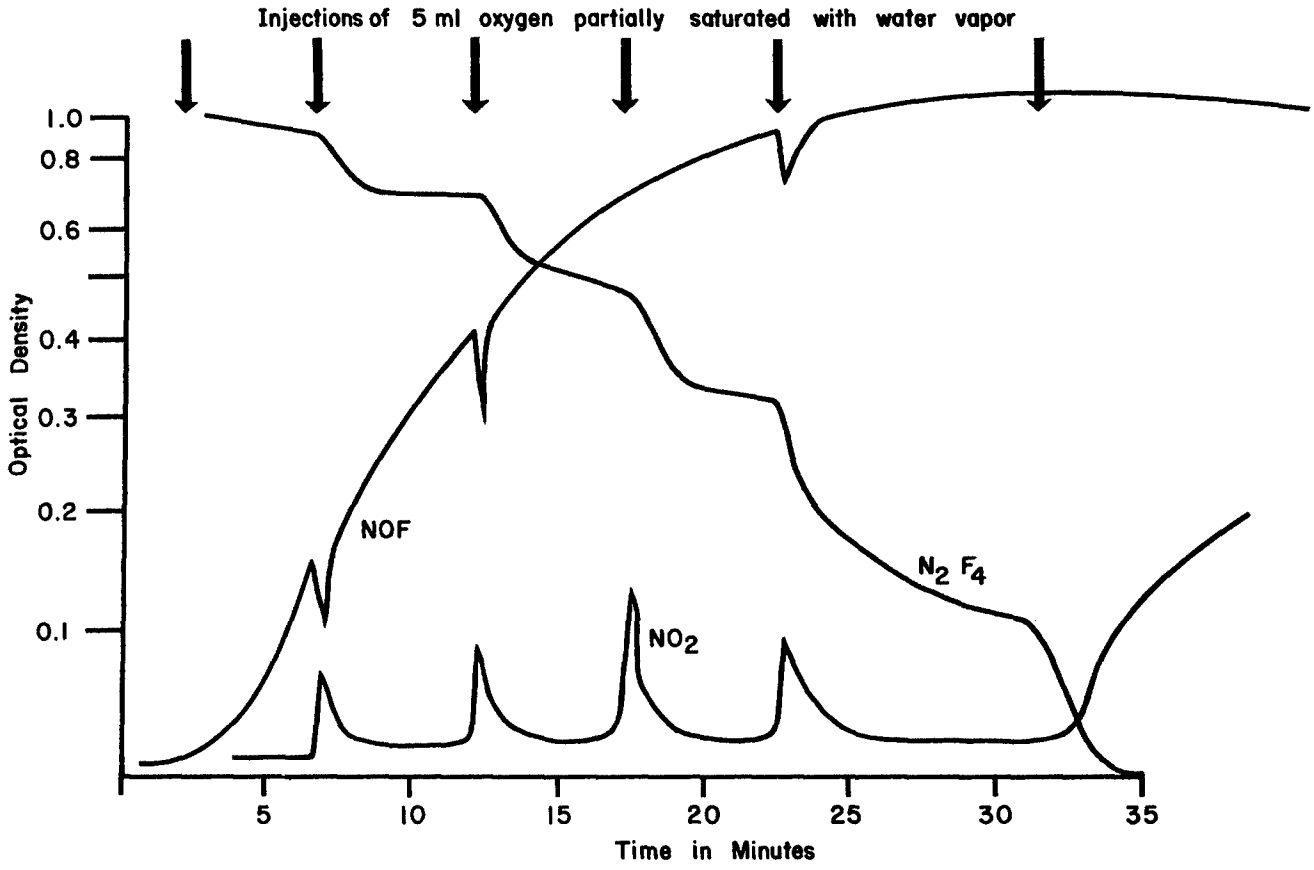

Figure 5. Effect of Oxygen Partially Saturated with Water Vapor Upon the Equilibrium Between NO_2 and NOF 23°C, 1 Atmosphere Initial Concentration of N_2F_4 2.7% in Nitrogen Cell: Teflon with AgCl Windows, 10cm optical path, total volume 75 ml

reaction of N_2F_4 has proceeded in an excess of water, substantial amounts of NO_2 may be in solution. In most cases, therefore, the observed NO_2 concentration at termination of an N_2F_4 decomposition sequence is substantially less than the stoichiometrically calculated value.

N_2F_4, on the other hand, remains intact for extended periods in contact with oxygen-free water in the IR cell. We have stored N_2F_4 over water for periods as long as two weeks with no detectable change in its infrared spectrum between 2 and 16 μ.

NO_2 usually fails to appear in measurable amounts when N_2F_4 is still present. However, if sufficient water is present, the formation of NO_2 from NOF may progress faster than the reaction of NO_2 with $NF_2\cdot$ to form NOF. NO_2 will then temporarily increase at the expense of NOF. As shown in Figure 6, the NO_2 concentration subsequently falls until N_2F_4 has disappeared.

The NO formed in reaction (3) may be expected to react with oxygen to form NO_2 which would then react as shown above. The rate of reaction between NO and O_2 is concentration-dependent with respect to NO according to Elkins (ref. 23), and this observation has been qualitatively confirmed in our laboratory. In air, the reaction is very fast at high NO concentrations but slows with lower concentrations so that some NO may be expected to persist in the gas mixture for extended periods. The reaction rate of NO at several concentrations in air, calculated according to Elkins (ref. 24) is instructive:

PPM of NO in Air - v/v	PPM of NO_2 Formed/Minute
3200	2800
800	176
200	11
100	2.8

There is evidence to show that the reaction $NO + F_2 \rightarrow NOF + F$ takes place at room temperature (ref. 25), but because of the expected reaction (3) these reactions will not diminish the net availability of NO. Due to the very low molar absorption coefficient of NO, we could not measure the concentration of NO present in these reaction mixtures. However, on the basis of similar biological effects caused by N_2F_4 and NO to be described later, substantial amounts of NO apparently remain intact in N_2F_4 atmospheres.

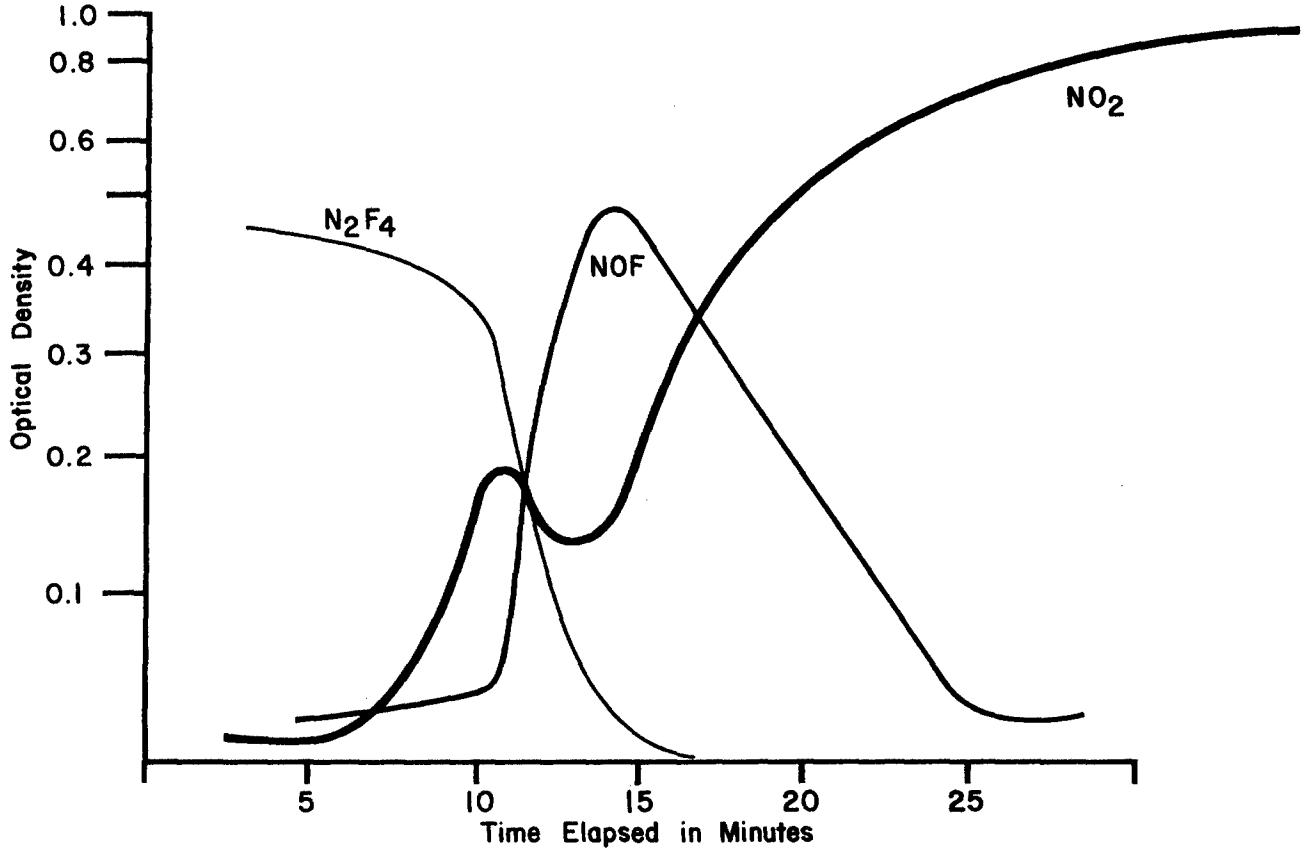

Figure 6. Time Course of N_2F_4 Decomposition in Water-Saturated Air
23^{o} C, 1 Atmosphere, Initial Concentration of N_2F_4 1.33%
Cell: Teflon with AgCl window 10 cm optical path, volume, 75 ml.

When all N_2F_4 has been converted to NOF, the final reaction is (3). In excess water, the terminal products will be nitric and nitrous acids:

$$2NO_2 + H_2O \rightarrow HNO_3 + HNO_2$$

HNO_2 decomposes in the manner:

$$2HNO_2 \rightarrow HNO_3 + H_2O + 2NO$$

At this point, the theoretical proposition made appears to be compatible with our experimental results.

Relation of Chemical Reactions of N_2F_4 to its Biological Effects

Two considerations have led us astray while evaluating the biological effects of N_2F_4. In early observations of sub-lethal intoxication, methemoglobin concentrations above 25% of total available hemoglobin in rat blood samples were not encountered, and, in most cases, these values were below 10%. Later re-examination of this parameter in lethally affected rats showed, however, that very high and potentially lethal levels of methemoglobin could, in fact, be caused by N_2F_4.

In examining the chemistry of N_2F_4, we have considered that the reaction of NO to NO_2 is relatively rapid and that no NO should be expected in any gas mixture containing oxygen. We have found, instead, that NO reacts with O_2 slowly at low concentrations, establishing that the presence of NO as well as NO_2 must be considered in judging the toxicity of N_2F_4.

Exposures to concentrations of NO_2 ranging from 0.002 to 0.1% for various periods brought to light an interesting and related fact: regardless of lethal effect, concentration, period of exposure or physical symptoms, NO_2 caused only a limited level of methemoglobin. In no animal of this series did conversion of hemoglobin to methemoglobin exceed 11%. NO, on the other hand, at concentrations of approximately 0.3%, caused methemoglobin formation at a rate somewhat faster than did N_2F_4 at a concentration of 1%. In at least two test animals, methemoglobin concentrations reached 85% before reflex activity was lost. This level is far above survivable concentration and apparently increased to that extent during the short period when the animals were expending cellular oxygen reserves and going into oxygen debt to maintain cardiac and other activity. It is not known whether the concurrent presence of NO_2 in this gas mixture was a contributing factor. We have concluded from this evidence that the

primary lethal effect in acute N_2F_4 intoxication is failure of respiratory transport, due to methemoglobin formation resulting from NO (nitric oxide) reaction with hemoglobin. There must be, in addition, substantial contribution from the known irritant effects of NO_2 and the corrosive damage done by HF. Nonetheless, almost all the animals that survive the first 10-15 minutes after exposure to N_2F_4 survive indefinitely, suggesting that in these animals primary damage is rapidly overcome, as is the case in a reducible methemoglobinemia.

In spite of the effective evidence for these conclusions, however, we have no certain explanation for the low levels of methemoglobin seen in our early experiments and also reported by Carson and Wilinski (ref. 11). The relation between those findings and the low methemoglobin concentration resulting from lethal intraperitoneal doses of N_2F_4 is also not clear, nor is the origin of the fluoride localized in erythrocytes after N_2F_4 inhalation. All of these discrepancies may depend upon the extent to which N_2F_4 decomposes in the toxic atmosphere by the time it reaches the interface with blood. If substantial N_2F_4 remains, the possibility for a number of known oxidizing species ($NF_2\cdot$, NO, NOF) to contact hemoglobin is increased. It is doubtful that NOF could remain intact, but NO and $NF_2\cdot$ may penetrate the cell wall. Some NO can be expected to remain intact in solution, and has been demonstrated as methemoglobin forming in our experiments. $NF_2\cdot$ remains as the only link between the chemical identities of N_2F_4 and NF_3 which corresponds with the burden of fluoride laid down in red blood cells by these two agents only. To maintain this relationship, it may be necessary to invoke a transient formation of $NF_2\cdot$ in the reduction of NF_3 by hemoglobin. Whether $NF_2\cdot$ then is responsible for oxidation of hemoglobin or subsequent reaction with methemoglobin is a matter for future study.

The role of NO_2, in spite of the relative lack of primary effect, may be quite important. The similar methemoglobin levels following NO_2 exposure at quite different levels is a manifestation which could be caused by an inhibition of the methemoglobin-reductase system at a site which is readily attacked, but which is responsible for only minor interference even when completely saturated. At higher levels of methemoglobinemia, such partial failure may have great significance.

The reaction of NO with hemoglobin is classically considered not to cause methemoglobin formation. In vitro studies over the past 30 years have demonstrated the reaction between nitric oxide with both hemoglobin and methemoglobin and have established that under the anaerobic conditions necessary to formation of the NO-hemoglobin complex, NO does not cause the formation of methemoglobin (ref. 26,27,28,29).

These in vitro observations are not necessarily contradicted by the very clear evidence in our laboratory that a gas mixture at an atmosphere in which the toxic component is primarily nitric oxide causes massive formation of methemoglobin.

One obvious difference lies in the essential presence of oxygen in any study of intact animals. None of the reports of in vitro NO-hemoglobin reactions mentions work in which oxygen was present; if methemoglobin were formed in such a case, its significance may have been dismissed with the laboratory accident of allowing oxygen into the experiment. This factor may be responsible for an observation (ref. 30) that in blood of rats exposed to 10 ppm of NO, the electron spin resonance character of the NO - hemoglobin complex did not appear, although the authors suggested that this may have reflected a failure of NO to enter the cell. It is questionable in any case, whether a concentration of 10 ppm is sufficient to guarantee passage of any agent into erythrocytes.

CONCLUSIONS

The existing evidence indicates that the acute lethal effect of N_2F_4 inhalation may be due to methemoglobin formation. Certainly, methemoglobin formation to an extent supposed to be lethal does take place. There are other factors which interfere with this simple analysis, however. The most important is that lethal doses of N_2F_4 injected intraperitoneally do not cause extensive methemoglobin and yet are lethal at total doses which are less than the amount contacted by inhalation.

While we have shown that of the terminal products of N_2F_4 decomposition in moist air, only NO causes methemoglobin, it has not yet been possible to determine whether the minimum concentration of NO which will cause substantial methemoglobin is realistic in terms of the dynamics of a toxic N_2F_4 exposure.

The limitation on the ability of our system to resolve the reaction rate of N_2F_4 in moist air has not yet permitted us to gather information enough to predict the extent of decomposition taking place during the brief respiratory cycle. Even with this information, prediction of NO concentration in the lung would be exceedingly complex; direct measurement would be impossible due to the optical absorption characteristics of NO. The possibilities of direct measurement of NO_2 as the

terminal product, coupled with uptake data for administered NO_2 would probably enable an estimate of the *in vivo* rate of decomposition.

NO_2 should not be dismissed as being an unimportant component of the toxic activity of N_2F_4. Its apparent ability to interfere with reduction of low concentrations of methemoglobin merits further study.

A further paradox exists as well. In our experiments, we have satisfied ourselves that in the environment expected in the lung, no fluoride bearing product other than HF should arise from the decomposition of N_2F_4. We have shown as well that such agents as ClF_3 and NaF, which may be considered related to HF as well as HF itself, cause no methemoglobin and deposit no residual fluoride in red blood cells. N_2F_4 does both, causing a localization of fluoride in red blood cells which persists at least 24 hours, and in these respects simulates the effect of NF_3. We have already stated that we have been unable to cause formation of NF_3 in observed decomposition reactions of N_2F_4. We have rejected the possibility that NOF is responsible because of its demonstrated, nearly instantaneous, reaction with water, but we have been unable to determine empirically whether our assumption is correct.

TOXICITY OF CHLORINE TRIFLUORIDE (ClF_3) AND BROMINE PENTAFLUORIDE (BrF_5)

INTRODUCTION

Much of the existing information on the chemistry of the interhalogens seems confined to their physical and chemical properties and use a fluorinating agents, and has been reviewed in a previous report (ref. 2). Remarks on reactions of interhalogens with water are usually limited to mention of the violently explosive consequences of such contact, although some speculation on these reactions has been presented (ref. 31). The first response, therefore, in considering the toxicity of interhalogens, has been to wonder whether these obviously reactive materials could remain intact long enough to exert primary effects on mammals or other biological entities.

In our related studies of environmental contamination by chlorine trifluoride (ClF_3) and bromine pentafluoride (BrF_5), reactions with water have been found to be immediate and extensive, as expected (ref. 31). There are apparently a number of products formed, including HF and oxidizing compounds of moderate vapor pressure and stability. At least some of the latter retain their oxidizing capability and biological activity for months in non-ventilated solutions. However, only a few of the products of interhalogen reactions with water have been identified in our experiments and these have been found relatively non-toxic to fish and microorganisms. Among the products yet to be identified, however, are compound(s) of very high toxicity to aquatic species. Whether these may also exert toxic effects following absorption across the mammalian pulmonary membrane in subacute exposure with limited lung damage, seems a potentially important question.

As with other compounds, it has been necessary to determine whether the acute toxicity of interhalogens is systemic or related to local damage at the site of contact. The limited existing information on interhalogen toxicity describes subacute and chronic experiments in which exposure conditions and materials incompatibility may have not permitted proper definition of exposure concentrations. It has been quite clear, however, that whatever the conditions, these exposure experiments and those conducted in this laboratory have indicated that interhalogens cause serious contact damage to mucous membranes and exposed skin and presumably to the respiratory membranes. The evidence available suggests strongly that almost

the entire effect of acute lethal exposure to interhalogens is by destructive reactions upon contact with the pulmonary membranes.

Our effort, therefore, has been focused on evaluation of the capability of the respiratory gas exchange mechanism, with a survey of some other physiological activities.

METHODS

Procedures used in common with other gases are discussed in the section on General Methods.

Transfer Syringe for Injection of Interhalogens

Efforts to inject pure gaseous ClF_3 intraperitoneally were complicated by the reactivity of the compound with glass, plastics and fluorocarbon oils. To facilitate direct transfer of the gas, a 5 ml syringe was constructed entirely of Teflon in the instrument shop of the OSU Physics Department (Figure 7). The barrel of the syringe is a through-bored 1-1/2" Teflon rod. The plunger was machined to fit after being tightly sleeved over a stainless steel core. The barrel is closed by a machined plug which bears a tapered male fitting for standard Luer needle attachment. Gas ports in the side of the barrel enable flushing of the syringe and transfer of aliquots from slowing gas streams. A guide-rod and calibration plate on the outside of the barrel indicate the position of the plunger. This syringe has proven satisfactory for gas transfers requiring limited precision. The cold flow characteristics of the Teflon barrel and plunger create changes in the barrel to plunger sealing and small quantities of gases are occasionally lost.

For most uses, a Hamilton 3-way Teflon valve is attached to the tip of the syringe to facilitate flushing and loading. For injection of ClF_3, the syringe is attached to the gas source by a flexible Teflon tube and flushed with several volumes of the gas. The valve is then directed through the attached 26 gauge x 1/2" needle and volumes up to 3 ml may be injected.

No attempts were made to introduce BrF_5 in this fashion since it could not be maintained in the vapor state; the use of liquid BrF_5 was precluded on the basis of its violent reactivity with water. When solutions of BrF_5 are made by direct introduction of BrF_5 liquid into water contained in polyethylene bottles, the reaction at the point of contact with water is audible with visible flaming in the water unless the mixture is made very slowly.

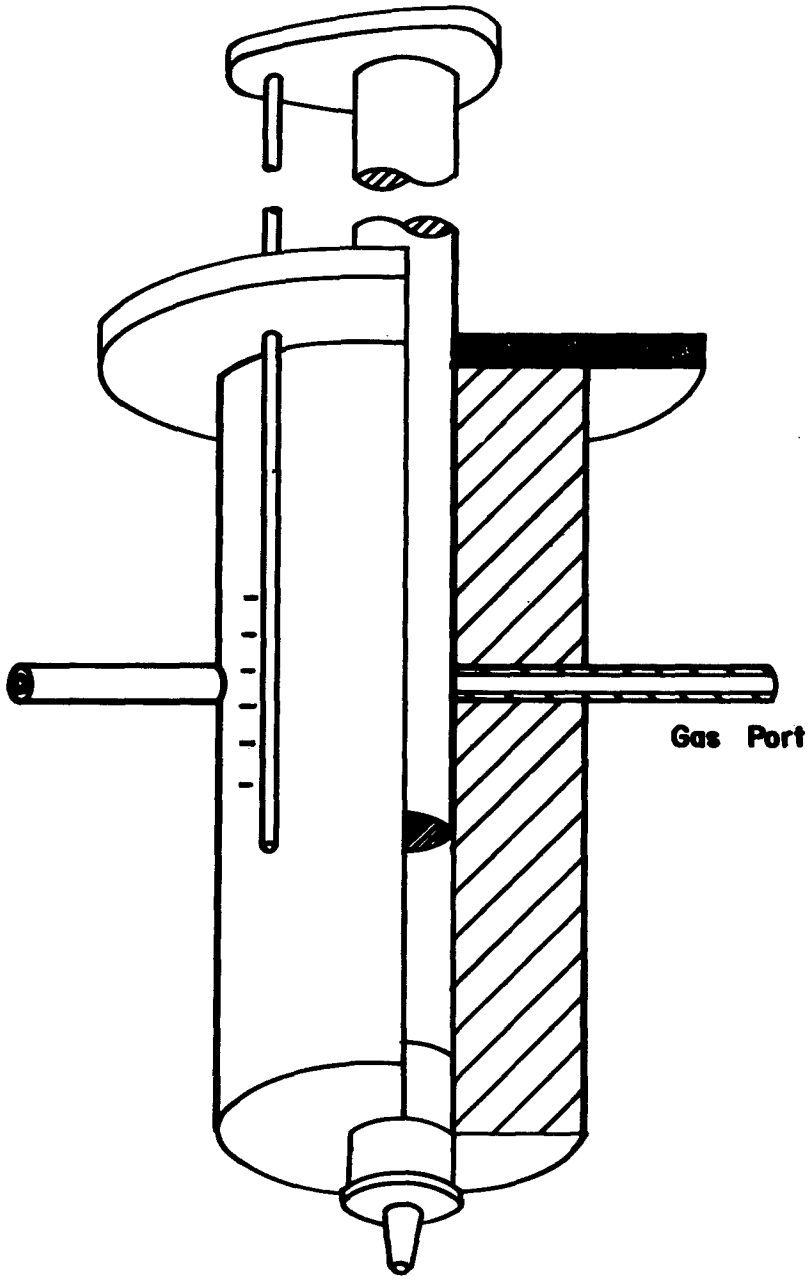

Figure 7. All-Teflon Syringe for Handling Undiluted Interhalogen Gases

Determination of Blood pH Following Exposure to Interhalogens

Measurement of blood pH was made in a vessel constructed to closely enclose each electrode of a Corning pH meter so that the total volume may be minimized. The vessel has two compartments, each constructed of the lower 3 centimeters of a 10-milliliter disposable syringe. The two chambers are connected by 3 cm of 3/32" Tygon tubing, penetrated midway between the chambers by a 0.2 millimeter diameter Teflon needle with Luer hub. Electrodes of the pH meter are fitted with gaskets which snugly insert into the halves of the vessel providing a gas-tight seal. Each chamber is vented below the gasket through a small hole which can be sealed by an elastic band.

Prior to measurement, the vessel is purged with nitrogen admitted through the Teflon needle. Three ml of cardiac blood, lightly heparinized, is injected by the same route, filling the chambers equally until the venting point is reached. Care is taken that negative pressure and head space within the syringe are minimized during wighdrawal. Unless already moribund, the rats are anesthetized with sodium pentobarbital to facilitate the withdrawal of blood. Such anesthesia has no effect upon blood pH of normal animals, even when depressed to a state of respiratory failure.

Determination of Urinary pH Following Exposure to Interhalogens

Preparation of animals for collection of urine was carried out by the same procedure employed for urinary fluoride analysis. To minimize the volume requirements in these studies, pH was determined in the manner described for the determination of blood pH.

Radiorespirometric Measurement of $^{14}CO_2$ Transport Across the Lung

Movement of $^{14}CO_2$ outward across the lung after intraperitoneal administration of $NaH^{14}CO_3$ was measured radiorespirometrically (ref. 32). Relative output of CO_2 before and after exposure was measured by infrared analysis. For each experiment, the intraperitoneal dose of sodium bicarbonate-^{14}C was 0.14 microcuries per animal and the chemical level was 32 milligrams per kilogram body weight. The time course of $^{14}CO_2$ evolution and the average rate of total CO_2 output by normal animals administered $NaH^{14}CO_3$ was measured, and after exposure of the same animals to ClF_3 and BrF_5, these measurements were repeated. Since each rat served as its own control, the cumulative $^{14}CO_2$ production of each intoxicated animal was expressed as the percentage of the cumulative CO_2 production prior to intoxication.

Effects of Ingestion of Interhalogen Solutions

Interhalogen solutions for ingestion by rats were prepared by slow direct injection of the gases into inverted polyethylene bottles filled with water. Teflon tubes with stainless steel needles are inserted directly through the vessel wall for introduction of interhalogens and for relief of pressure if gases are formed over the solution. The resulting stock solutions of ClF_3 and BrF_5 were diluted to 100 micrograms of fluoride per milliliter and each was divided in half.

One volume of each agent was brought to pH 6.5 by addition of sodium bicarbonate and the other was used at its original pH of 2.9. Four rats were maintained on each of these solutions as the sole source of drinking water for 10 days. The only concern in these observations was to determine whether acute toxicity of any kind might be induced by products of the reaction of interhalogens with water.

RESULTS AND DISCUSSION

Symptoms and Lethality of ClF_3 and BrF_5

Exposure of rats to ClF_3 initially caused a severe mucosal irritation in which all of the exposed tissues were severely inflamed, accompanied by substantial lacrimal discharge. Early in high concentration exposures and throughout low concentrations of ClF_3, the animals preened themselves excessively and exhibited no apparent depression. With increased time or concentration, all surface damage became more pronounced and soon after exposures began, the hair became brittle and yellowed. Corneal opacity often appeared. The respiration was very shallow without appreciable increase in rate.

Exposure to 0.08% ClF_3 (800 ppm) for 15 minutes resulted in death in almost every instance, but 12 or 13 minute exposures were seldom lethal. An atmosphere of 400 ppm was lethal in almost every case after 35 minutes of contact (Table XI).

We have observed that almost all rats able to survive 4 hours after exposure to ClF_3 in air will survive indefinitely. Intoxication of animals with ClF_3 and BrF_5 even for short periods should be considered potentially lethal, however, since the afflicted rats often became incapable of normal functions. Corneal and conjunctival damage interferes with vision; the animals cease to eat for several days and presumably are

TABLE XI

Lethality to Rats of Inhaled ClF_3

Exposure Period (minutes)	0.08% (800 ppm) ClF_3 dead/total exposed	Survival of Lethally Intoxicated Rats (minutes)
30	4/4	20 - 160
25	6/6	20 - 100
20	8/8	20 - 45
15	10/10	45 - 90
13	1/8	90
10	0/10	-----
	0.04% (400 ppm) ClF_3	
40	8/8	55 - 110
35	7/9	60 - 100
30	4/6	90 - 140
25	0/4	(1 rat died at 7 hours)
20	0/8	

TABLE XII

Lethality to Rats of Inhaled BrF_5

Exposed Period (minutes)	0.1% (1,000 ppm) BrF_5 dead/total exposed	Survival of Lethally Intoxicated Rats (hours)
20	0/10	
25	12/12	0 - 45
	0.05% (500 ppm) BrF_5	
40	0/10	
50	11/14	0 - 10

susceptible to infection introduced across damaged membranes.

A few animals were administered gaseous ClF_3 intraperitoneally in order to determine whether the effective dose was similar to the amount of ClF_3 presumed to be contacted during an inhalation exposure. Assuming that a 300 gram rat ventilates at the rate of 0.1 liter per minute, during a period of 15 minutes in an atmosphere of 0.08% ClF_3 he will contact no more than 45 micromoles of ClF_3. The decreased ventilation observed in all inhalation exposures must considerably lower this figure. We have not been able to cause lethal effects with intraperitoneal doses of up to 3 ml (130 μmole) undiluted ClF_3. Because of the extreme discomfort to test animals and the violent reactivity of ClF_3 when administered intraperitoneally, doses larger than this were not given. This comparison suggests that systemic effects of ClF_3 or its products following absorption across the lung are not the primary cause of death in acute intoxication. The fact that contact destruction resulting from intraperitoneal ClF_3 injection is non-lethal relates to the non-essential role of the peritoneal membrane in the moment by moment activities necessary to survival. Similar damage in the lung must be certainly expected to jeopardize life, and since the lung is the area of contact in exposure to volatile ClF_3, it is reasonable to expect impairment of pulmonary function in intoxicated animals.

The effects of BrF_5 are, in general, similar to those of ClF_3; but the animals affected appear to suffer more severe gross damage to the mucous membranes. The hair is more severely attacked, often becoming quite sticky and there is evidence of considerable yellow streaking on fur, which may be deposition of free bromine. Even in sublethal exposures, there may be severe necrotic damage to the feet and other areas of skin which are not adequately protected. As in the case of ClF_3, exposed animals refuse food for several days following sublethal exposure.

At 0.1% BrF_5 in air, no exposure of less than 20 minutes was lethal; no exposure over 25 minutes was survived (Table XI). At 500 ppm, a 50-minute exposure is usually lethal although exposures of 40 minutes or less do not cause death.

Substitution of aqueous solutions of ClF_3 and BrF_5 at concentrations up to 100 micrograms fluoride/ml for normal drinking water for two weeks was found to be non-lethal. These preparations were administered as buffered and unbuffered solutions. The animals so treated did not gain weight normally and were generally poor in appearance, but it was quite clear that by this route of administration, the aqueous reaction products of ClF_3 and BrF_5 were not acutely toxic.

Clinical Chemistry

The results of measurements of blood constituents in animals exposed to the interhalogens are clinically of little use. A transitory increase in serum α-1-globulin appeared and then regressed during the first few hours after BrF_5 exposure (Table IX). Blood glucose was elevated immediately following exposure to both agents, but also returned to normal levels within a few hours (Table IX). Animals exposed to BrF_5 responded to a lesser extent than to ClF_3. The qualitatively similar effect of each of the agents on blood glucose may represent adrenal medullary discharge in a non-specific response to irritation. The effects of ether anesthesia on this parameter are exhibited in the control animals but are far exceeded by the effects of the toxic gases (Table IX).

Neither ClF_3 nor BrF_5 caused measurable methemoglobin formation in rats at any level of exposure. It is known that NF_3 and N_2F_4 intoxication is accompanied by probably lethal methemoglobinemia, which may be considered evidence of the survival of either the original agents or strongly oxidizing products of reactions of the original agents. Inability of the interhalogens to produce similar effects may be interpreted as either failure to reach hemoglobin in a reactive form, or less likely, inability to oxidize the heme iron. Coupled with the evidence that only a small amount of fluoride is found in red cells after exposure to interhalogens and that persistent high concentrations are found after exposure to nitrogen fluorides, it appears reasonable to suggest that ClF_3 and BrF_5, as such, fail to survive contact with lung tissues and do not reach the circulation in a highly reactive form.

Effects of ClF_3 and BrF_5 on Respiratory Gas Transport

The reactions of ClF_3 in an aqueous system proceed very readily to form inorganic acids, and highly reactive oxidizing species.

There is no reason to expect that similar reaction products are not produced in a dilute gas atmosphere of ClF_3 as it contacts atmospheric water vapor and the water-saturated respiratory gases. These products, especially HF, should be expected to cause extensive contact damage, and, in fact, the upper respiratory tract of interhalogen exposed animals vividly shows evidence of such activity.

These effects occurring in the lung should be reflected in obstructed gas transport. The pulmonary damage expected here may not necessarily cause a net decrease in steady-state CO_2 evolution, but by

increasing the barrier to diffusion will increase the partial pressure gradient between the circulation and the atmosphere. Any fixed amount of CO_2 will dilute in the resultant larger carbon dioxide-bicarbonate pool of intoxicated animals and should accordingly be released at a slower rate than from normal animals. Experimentally, this is the case. When sodium bicarbonate-^{14}C is administered to interhalogen intoxicated rats, a distinct change from the normal pattern of $^{14}CO_2$ evolution is seen.

A healthy rat produces a rapid initial output of $^{14}CO_2$ during the first 20 minutes of an experiment, after which the rate gradually declines as the administered label becomes integrated with endogenous bicarbonate. Interhalogen intoxicated rats show marked effects during this early period as can be seen when comparing observations of individual rats before and after intoxication (Table XIII).

Total CO_2 output may be somewhat lessened during these experiments, particularly during the phase of acute failure just prior to death. The generally lowered CO_2 output probably results from decreased metabolic activity resulting from the quiescent behavior of interhalogen affected animals. Similar changes can be observed in animals severely depressed by deep anesthesia, and should not be considered directly related to metabolic impairment by the interhalogens.

Effect of ClF_3 and BrF_5 on Blood and Urine pH

Measurement of blood pH after ClF_3 or BrF_5 intoxication has led to some confusion. Interference with CO_2 evolution should be expected to cause a general acidosis; as expected, blood pH is markedly lowered by ClF_3 intoxication. For reasons which we have not established, BrF_5 has much less effect (Table XIV). Urinary pH is depressed below control levels by ClF_3 for at least 48 hours but does not fall below the nominally normal range. Exposure of animals to BrF_5 did not noticeably affect urine pH. It is highly doubtful that absorbed acids are responsible for these effects. The amounts of gases contacted are not sufficient and the measured fluoride in blood is far less than is necessary to account for such changes.

There is as yet no explanation for the difference in behavior of the two agents. The possibility of experimental error has been examined by repetition of the experiments under different levels of exposure by different personnel.

The imbalance causing these manifestations is somewhat self-limiting, since lowered pH tends to expedite the liberation of CO_2 from

TABLE XIII

Effect of Interhalogen Intoxication Upon Release of $^{14}CO_2$ Administered Intraperitoneally as $NaH^{14}CO_3$ to Rats

$$\frac{^{14}CO_2 \text{ After Intoxication}}{^{14}CO_2 \text{ Before Intoxication}} \times 100^*$$

BrF_5 0.05% in air, 40 minute exposure

Time (Minutes)	Animal 1	2	3	4
10	105	4	5	41
20	45	26	18	22
30	64	42	30	53
40	67	54	39	63
50	69	62	47	68
60	71	70	55	70
120	72	86	82	73
240	71	95	91	75

ClF_3 0.04% in air, 20 minute exposure

Time (Minutes)	Animal 1	2	3	4
10	55	29	8	17
20	53	41	15	37
30	57	48	23	55
40	63	55	30	66
50	67	61	37	73
60	72	66	43	76
120	75	80	63	76
240	76	86	76	76
360	75	88	75	76

*Percentage of cumulative $^{14}CO_2$ production by same rats prior to intoxication

TABLE XIV

Effect of Interhalogens on Blood pH

Treatment	Duration of Exposure (minutes)	Sample Time After Exposure (Minutes)	pH Measured
Control, light anesthesia			7.56
			7.73
			7.58
			7.51
			7.59 Average
Control, deep anesthesia			7.63
			7.52
			7.57 Average
ClF_3 0.08% (light anesthesia)	15	20	7.00
	15	20	6.91
	20	15	6.88
	20	25	6.78
	30	Immediately	6.74
	30	"	7.04
	25	"	6.97
	25	"	7.10
			6.92 Average
BrF_5 0.05%	50	"	7.31
	50	"	7.40
	50	"	7.30
	50	"	7.38
	50	"	7.27
	50	"	7.42
	50	"	7.46
	50	"	7.30
			7.35 Average

the blood, and the increased partial pressure of CO_2 tends also to overcome the obstruction to the diffusion process.

These effects upon gas transport are not caused by severe depression. The pH of blood drawn from rats anesthetized to the point of failing respiration with pentobarbital sodium or ether exhibit none of the changes in blood pH or $^{14}CO_2$ release which are seen in ClF_3 intoxicated animals.

CONCLUSIONS

There seems little indication from the evidence we have obtained that systemic effects of ClF_3 or BrF_5 are responsible for their acute lethal effects. The failure of large doses of intraperitoneal ClF_3 to cause more than severe local discomfort is particularly significant, as is the failure to oxidize hemoglobin. Distribution and behavior of fluoride ion originating with interhalogen exposures is similar to that after administration of sodium fluoride, suggesting a non-specific effect. Of greater significance is the decreased respiratory effectiveness, measured directly and in terms of blood and urine pH changes, which argue strongly that the acute effects are due to local contact damage of the respiratory tract.

At the same time, the demonstrated stability of some products of the reaction of ClF_3 and BrF_5 with water must not be ignored when considering sub-acute exposure.

TOXICITY OF OXYGEN DIFLUORIDE (OF_2)

INTRODUCTION

The existing literature indicates that the toxicity of OF_2 is vastly greater than any other compound with which this program is concerned. A survey of literature by the Advisory Center on Toxicology quotes two reports in which the median lethal concentration time product for OF_2 (ref. 33) was stated to be 150 mg/cubic meter/minute or 63 ppm /minute. Lester and Adams indicate that respiratory contact with 10 ppm for 10 minutes was lethal to rats (ref. 34). LaBelle, et al., found that some rats would live for as long as 1.5 hours in an atmosphere of 10 ppm (ref. 35).

Pùlmonary pathology studies by Lester and Adams showed that immediate effects were minimal; damage of toxic significance did not appear in the lungs of lethally exposed rats for many hours. Some systemic effects were detectable in these animals but were not sufficient to cause death or serious symptoms (ref. 34). The studies of LaBelle (ref. 35) and of Cianko (ref. 36), while less extensive than those of Lester and Adams, indicated similar effects. These findings indicate that OF_2 is not directly corrosive; its toxic effects in the lung are not related to its reactions with the surface of the pulmonary membrane.

OF_2 is fairly stable in aqueous systems (ref. 37) and should be expected to survive chemically in the atmosphere of the lung and in the gas transport processes without immediate reaction on pulmonary surfaces. It is soluble enough (6.8 ml OF_2/liter of water at 0^o) to permit transport into cells in the low amounts evidently necessary for lethal effects. We have found in related studies (ref. 31) that OF_2 degasses quickly from aqueous solution when its partial pressure in the atmosphere falls. In the course of an exposure, the gas should therefore be present in the lung only during the period of exposure, leaving a minor amount of HF resulting from the limited reaction of OF_2 with the aqueous phase of the pulmonary membrane complex.

Our animal studies have, in general, supported the observations derived from those made previously and considered with them suggest that OF_2 reacts within the pulmonary cells after a non-destructive transit across the cell membrane from the alveolar space.

METHODS

Procedures used in the study of OF_2 intoxication have been described or identified in the General Methods section.

RESULTS AND DISCUSSION

Symptoms and Lethality

The principal visible symptom of OF_2 intoxication at all concentrations including those low enough that survival seems assured, is an accelerated shallow respiration at about 150 cycles/minute. On palpation, the costal elevation seems to pulse or throb. This abnormal respiratory activity is usually accompanied by a forward thrusting of the head. These symptoms are apparent immediately upon removal of rats from the exposure chamber and may persist until death or through several days of recovery. The animals often appear to be in pain, and stay in a huddled position, only occasionally engaging in some stretching movements. A few hours after exposure, the hair coat of all animals is very ruffled. Whether this is a primary effect or merely due to lack of grooming is not known. The more severely exposed animals appear unusually susceptible to stress, and if handled or allowed to run freely, may die within a short time following exertion.

There is some darkening of the eyes and extremities which is suggestive of faulty respiratory exchange, possibly as a result of congestive pulmonary disturbances. Elevation in methemoglobin levels is not responsible for these changes, since the concentration of methemoglobin in animals exposed for 15 minutes to 15 ppm of OF_2 constitutes no more than 1-3% of the total hemoglobin concentration. At the higher exposure levels, there are often copious amounts of clear serous discharge from the nasal passages and mouth, and there is also occasional reddening and slight bleeding in the upper respiratory tract.

The lethal concentrations which we have observed for OF_2 seem somewhat higher than other investigators have reported, but at the low concentrations for lethal effect of this compound, such differences probably have no significance. The relation between the duration of exposures to 15 and 30 ppm OF_2 and the time elapsed between termination of these exposures and the death of each rat is illustrated in Table XV. Experiments in which animals of different weight ranges were used are shown separately.

TABLE XV

Lethality of 15 and 30 ppm (v/v) OF_2 to Rats at Two Weight Ranges

		Minutes of Exposure			
		10	15	22.5	34
I.	350-400 grams, 15 ppm	2.0*	8.1	4.6	0.2
		9.1	10	4.3	0.4
		18	29	5.2	0.9
		19	30	5.7	1.4
		24	32	9.5	1.7
		37	34	12	2.7
			42	13	3.0
		(13 rats)**			
			48	15	3.1
				15	3.9
			(2 rats)**	18	5.7
II.	490-550 grams, 15 ppm		12	2.8	0.7
			13	6.5	1.7
			15	7.7	2.4
			22	16	2.7
			30	19	3.1
			31	23	3.5
			36	24	3.6
			47	26	4.2
			48	35	5.0
			49	37	23
III.	490-550 grams, 30 ppm	3.0	2.8	0.3	
		3.6	4.3	0.3	
		3.8	5.0	1.4	
		4.0	5.2	3.6	

*Each figure represents survival time in hours following exposures
**Survived 5 days and apparently recovered

This factor apparently has limited influence on lethality.

A compound as stable and as lethal upon inhalation as OF_2 should have substantial toxicity when it is administered by other routes. This appears not to be the case, however, since we have been unable to cause symptoms or death from doses as high as 1 millimole/kilogram injected intraperitoneally as OF_2 gas. It would appear, therefore, that the gas reacted within or upon the first cells that it contacts without reaching the circulation. If the area damaged is not acutely vital to the animal, no serious symptomatic effect appears. We have examined the gross pathology at the site of intraperitoneal injection of OF_2 and find a course of effect that is similar to that which appears to prevail in the lung. During the initial two hours after the injection, little evidence of injury can be seen other than needle wounds and a few petechial hemorrhages on the viscera. By 6 hours, there is further evidence of hemorrhage, some adhesions, and moderate necrosis. By 16 hours after injection of the gas, the affected areas were seriously necrotic and by 30 hours, much of the damage appeared to be organized and under repair. One injection which was misplaced into the extraperitoneal space adjacent to the kidney caused considerable local destruction of the peritoneal membrane. There was no gross evidence of distress after intraperitoneal injection of OF_2, except in two rats exhibiting the respiratory movements characteristic of inhalation exposures.

Effects of OF_2 Upon Blood Chemistry

Clinical chemistry observations show little change over a 20-hour period following OF_2 exposure for 10 minutes at 15 ppm (v/v). This dose is high enough that a few animals of the series observed died before they could be sampled. Blood glucose rises moderately, and remains somewhat higher than normal for the entire period of observation in contrast to findings following exposure to other agents. While measurements of blood urea nitrogen and serum creatinine showed little departure from normal, the α-1-globulin fraction was elevated throughout much of the period of observation (Table IX). It is possible that this change reflects the moderate renal damage described by Lester and Adams (ref. 34).

Effects of OF_2 Upon Integrity of Erythrocytes In Vitro

The prolonged delay between exposure and appearance of pulmonary injury and the relative stability of OF_2 indicate that OF_2 moves into pulmonary cells as OF_2 and then either reacts with intracellular reducing systems or slowly forms HF, to cause lethal damage to metabolic mechanisms within the cell. Cells injured in this manner should retain structural integrity for

some time after initial metabolic disturbance, and should continue to serve their essential function as a diffusion membrane for respiratory gases until physical failure of the cell.

One test of this hypothesis made use of erythrocytes as models for pulmonary cells. The intact red blood cells were subjected to varying concentrations of OF_2, and the physical integrity of the cells was observed for a period following exposure.

The cells, as used, hemolyzed much more readily under the effects of OF_2 than when exposed to NF_3 as a comparison, but it was necessary nevertheless, to use such extensive exposures that no valid conclusion could be drawn. It is possible, however, that erythrocytes, as specialized cells with unique function, are not as sensitive to the metabolic lesions induced by OF_2 and are therefore an unsuitable experimental preparation.

In the period following exposure to NF_3, N_2F_4 and the interhalogens, there appears to be a generally uniform distribution of fluoride throughout the body. In each case there are certain tissues which appear to selectively accumulate more fluoride, but in none of these exposures does the lung, which is the organ of contact with each agent, become unusually laden with fluoride. This is not surprising, since NF_3 appears to pass intact through the pulmonary membrane, and the fluoride originating in N_2F_4 and the interhalogens appears at the respiratory surface as hydrogen fluoride, which should be rapidly removed as fluoride ion.

If the effects of OF_2 upon the lung depend on reaction within pulmonary cells and subsequent cell death, fluoride ion originating in OF_2 may remain in the lung for some time. The reducing environment of cell components should react with OF_2 readily, preventing passage of OF_2 as such out of the cell into the circulation for more general distribution. The fluoride released in this process should exchange passively into the circulation, since structural integrity of cells is maintained even if lethal metabolic damage has already been incurred.

Fluoride analysis of lung tissue was attempted in spite of the very limited amount of fluoride contacted during a minimum lethal exposure to OF_2. The difficulty may be appreciated when it is recalled that a rat exposed to 15 ppm OF_2 for 10 minutes contacts about 25 μg fluoride, less than 0.1 μg/gm. If a demonstrable increase in lung fluoride could be detected, even if it were too slight to be accurately measured, additional support for the general hypothesis might be provided.

The analyses, when made, suggested that more fluoride is present in the lungs of OF_2 intoxicated rats than in normal animals, but the differences observed are within the limits of experimental error and cannot be statistically validated.

A further argument in favor of local biochemical effects in the lungs is found in the data of LaBelle, et al., who showed that rats can survive up to 90 minutes of continuous exposure to an atmosphere of 10 ppm OF_2. A 15-minute exposure at this concentration will cause death after a delay of a few hours. This means that there is such limited local irritant effect that the animals can tolerate many times the ultimately lethal dose without respiratory impairment.

CONCLUSIONS

It is obvious from the description of OF_2 pathology in the literature, especially the work of Lester and Adams, (ref. 34) that OF_2 is not a corrosive agent. In other words, the contact and reaction of the agent at the surface of the pulmonary barrier does not immediately destroy the cell structure. The chemistry of the agent is compatible with this behavior as well. Limited reactivity, and limited, but real solubility indicates that the agent can remain intact in the aqueous pulmonary environment. Because it is not an ionizable compound, the barriers associated with charge differences should not impede movement into pulmonary cells.

The requirements for chemical activation and reduction of OF_2 that would at the same time result in metabolic injury within the cell are quite reasonably met in pulmonary cells (or other cells of contact). Experience in this laboratory with NF_3, which is less reactive than OF_2, shows that mechanisms within the erythrocyte are readily available for such reduction. The limited damage found in organs and tissues other than the lung, indicates that this agent has not achieved more than limited distribution, but has reacted primarily in the pulmonary cells. Whether the damage seen in other tissues (ref. 34,35) is primary to the effect of the agent, or secondary to lung damage is not established.

SECTION IV

DISTRIBUTION OF FLUORIDE ION IN ANIMALS INTOXICATED WITH INORGANIC FLUORIDE OXIDIAING AGENTS

INTRODUCTION

The correlation of data on fluoride distribution in animal tissues with the behavior of inorganic fluoride oxidizing agents or their products during intoxication is subject to considerable uncertainty. The observed fluoride content may represent the distribution of the parent compound, its initial reaction products or the fluoride ion resulting from the ultimate degradation of the compound. In addition, it is almost impossible to estimate the total amount of fluoride accumulated during intoxication because bone serves as a rapidly acting depository for fluoride ion, and as a consequence, fluoride levels in bone vary by amounts greater than the total fluoride content of soft tissue. This is unfortunate, because it would be of value to know whether a given agent is scavenged from the toxic atmosphere completely, or whether only a small fraction of it actually enters the animal. Measurement of the rate of release of fluoride into the urine of intoxicated animals was also found to provide little information. In spite of these limitations, the measurement of fluoride concentrations of tissues and in the contents of the digestive tract has provided useful information.

When this program was initiated, it was expected that detection of very small differences in tissue fluoride would be necessary in order to use this parameter as a source of information. In order to more easily define increments due to intoxication, an attempt was made to lower and possibly stabilize tissue fluoride levels by decreasing the fluoride intake of very young animals over a period of active growth. It was further hoped that the substantial variability found to exist among similarly treater normal animals could be controlled by establishing a low stable fluoride intake.

Tissue levels of fluoride were indeed found to be lower in animals subjected to a low fluoride intake, but the approach was considered unsuccessful because fluoride distribution following intoxication of these animals was qualitatively different from that of intoxicated normally fed animals. Some of the data obtained is discussed in this report since it is of general interest despite its failure of application to this program. As information accumulated, it also became clear that the changes seen

in intoxicated normally fed animals were of significant magnitude to allow proper study of fluoride distribution under toxic conditions.

The observation of changes in tissue fluoride concentration during the period following acute intoxication has been of the greatest value. Perhaps the most important finding has shown that the fluoride burden added during intoxication by each agent tends to leave almost all tissues simultaneously, making it possible in each case to infer behavior analogous to that of fluoride ion. The most marked exception is the very high retention of fluoride in erythrocytes of rats intoxicated with nitrogen fluorides, which has provided valuable insight into the mechanisms by which these intoxicants act. Observation of change in fluoride concentrations of visceral contents during the period following intoxication has proved of interest in evaluating excretion mechanisms.

METHODOLOGY

Measurement of Fluoride Content of Biological Material

Methods used in fluoride analysis have been described in detail in a previous documentary report based on efforts during the first year under this contract (ref. 38). The technique employed in routine analyses has been based upon that of Weinstein, et al., with slight alterations in which the Technicon Autoanalyzer performs all operations following sample ashing (ref. 39).

The sensitivity of this method is such that the fluoride content of samples must range between 1 to 100 μg. Some other procedures are capable of measuring smaller amounts, but the material customarily analyzed in this program contains enough fluoride that higher sensitivity is rarely required.

When necessary, the more tedious method of Hall (ref. 40) was used for analysis of samples containing between 0.1 and 1 microgram total fluoride. Comparison of the two methods in appropriately diluted samples has established that the results obtained with the two procedures agree very well.

At the maximum rate of operation of the Autoanalyzer, 10 ashed samples can be analyzed per hour. In practice, two technicians can process a maximum of only about 30 samples per day due to the tedious preparation of tissues for analysis. The procedures for cleaning crucibles

properly are also time consuming and require careful supervision. The same extensive sample preparation also accompanies the Hall method of analysis.

A specific limitation in this analytical procedure lies in an interference phenomenon associated with the high ratio of chloride to total ash found in plasma. Treatment of samples containing substantial chloride with perchloric and sulfuric acids as in the procedure used results in formation of HCl which volatilizes and passes through the system into the indicator mixture where it exerts a bleaching effect to cause a negative or decreased response. This effect cannot be detected in most tissues, but in highly vascular organs, such as lung, from non-intoxicated animals which have not been exsanguinated, the normal fluoride level may be undetectable.

The error in blood samples from control animals is similarly constant. Nonetheless, the specific correction of about 2 μg F/gm plasma was not applied, since our purpose is not measurement of absolute values but observation of the change in fluoride content of tissues following intoxication. No information has been provided where plasma measurements fall below measurable levels. The only intoxicated animals where this effect was noted were treated with NF_3.

Preparation of Intoxicated Animals for Study of Fluoride Distribution in Tissues, Gastro-Intestinal Contents and Urine

Tissue fluoride distribution and the concentration of fluoride in the contents of the digestive tract after intoxication were determined in rats that had been exposed to the respective agents for half the time required to kill a majority of the animals at the concentration used. Animals were then anesthetized, bled, and sacrificed for dissection at intervals up to 48 hours after exposure.

Fluoride excretion in urine was observed in animals exposed to each agent except OF_2. Rats were maintained for 2 days before and 3 days after exposure on a diet of laboratory chow with 5% sucrose in drinking water to increase urine volume. Except during exposure, the animals were kept in simple funnel bottom metabolism cages. Urine was collected under toluene and frozen for future analysis whenever a measurable amount was accumulated.

Preparation of Digestive Tract Contents for Fluoride Measurement

The entire digestive tract of animals exposed to ClF_3, BrF_5, NF_3 and sodium fluoride was removed and the stomach, small intestine,

caecum and large intestine were isolated. In preparation for analysis, each organ was carefully opened, cleaned and rinsed. The solid contents and rinsings were combined, dried and weighed prior to assay for fluoride concentration.

Preparation of Biological Material for Analysis

Material analyzed was prepared in the following steps:

1. At the time of dissection, tissues were blotted and placed in small plastic bags for weighing and freezing. Blood, urine and digestive tract contents were stored in small glass vials with screw tops.

2. Tissue samples were thawed in nickel crucibles, with sufficient distilled water to cover and then chopped with scissors.

3. 1 ml of 0.3 M magnesium succinate solution (fluoride free) and 4 ml of 1 N lithium hydroxide (fluoride free) were added to disintegrate tissue and to complex fluoride ion during later ashing.

4. Samples were slowly dried under infrared lamps, then weighed.

5. Disintegrated tissue was ashed in covered crucibles at 500^{o} C for 8 hours, then the temperature was allowed to drop slowly at the cooling rate of the furnace.

6. Ash was then suspended and dissolved in 5 ml of 2 N perchloric acid and transferred quantitatively to the sample tube of the Autoanalyzer.

Urine, blood and digestive tract contents as well as tissues are routinely treated according to the procedure above. We have found also that bone may be assayed by this method of preparation and that the more extensive procedures originally employed are not needed.

Procedure for Maintenance of Rats on Limited Fluoride Intake

Fluoride concentration of standard Purine laboratory chow at the time these experiments were conducted was found to be 25 micrograms fluoride per gram. The low fluoride diet contained 3 micrograms fluoride per gram and was constituted of casein, 18%; cornstarch, 72%, corn oil, 6%; cod liver oil (Squibb), 0.5%; Nutritional Biochemical Corporation salt mixture #14, 1%; and a vitamin mixture, 1%. The vitamin mixture was prepared

in a starch filler as follows: Thiamine HCl, 300 mg, nicotinic acid, 5 gm; pyridoxine HCl, 300 mg; D-pantothenic acid (calcium salt) 2 gm; choline chloride, 200 gm; riboflavin, 600 mg; and ascorbic acid as an antioxidant, 100 gm and 691.9 grams starch. The recommended percentage of the salt mixture is 4% of the total diet. Because of the high fluoride content measured in the mixture, the amount was reduced to 1%. There was no indication during the experimental period of any deficiency effects.

Feed was available to these animals at all times in small pans bolted inside each cage. The powdery consistency of the preparation resulted in a high loss of feed, but most of the animals receiving this diet maintained a normal growth rate. The few rats that did not learn to use the powdered feed were not used in this series of experiments. No attempt was made to estimate the intake of fluoride because of the great number of animals involved, and the difficulty of measuring daily consumption of the powdered diet.

The data obtained by measuring fluoride concentration of various biological materials is not amenable to detailed statistical analysis because in most cases the numbers of observations available to define a single point are too few, and variation among samples is high. These difficulties were anticipated at the outset, since we expected that variability among observations of similar treatments would in many cases be extreme, and we knew that the time and labor cost of each sample was prohibitive to large numbers of animals. In spite of this limitation, the character of fluoride distribution during the several hours following intoxication has been clearly shown.

RESULTS AND DISCUSSION

Distribution of Fluoride in Tissues of Non-Intoxicated Rats Maintained on a Standard Laboratory Diet

Several groups of animals have been examined in order to determine the distribution of fluoride in organs and tissues of normal animals. One group of determinations was made to provide control data for studies of fluoride distribution in animals maintained on a fluoride-depleted diet (Table XVI). In later experiments, the tissue fluoride content of each of five normal animals was used to provide control data in observing the effects of the various inorganic fluoride intoxicants on tissue fluoride distribution. Two such groups which were separated in time by about 2 months demonstrate one of the problems to be contended with in this work (Table XVII).

TABLE XVI. Effect of Fluoride Depletion of Tissue Fluoride Concentrations in Non-Intoxicated Rats, and Rats Immediately Following Intoxication with 10,000 ppm NF_3 for 55 minutes

	Non-Intoxicated Rats		Following NF_3 Intoxication	
	Fluoride Depleted	Standard Diet	Fluoride Depleted	Standard Diet
	$\mu gF^-/gm$*	$\mu gF^-/gm$	$\mu gF^-/gm$	$\mu gF^-/gm$
Bone	173 (97 -213)8	308 (230 -403)6	241 (232 -261)4	258 (169 -327)6
Lung	0.5(0.2- 0.8)8	2.0(0.8- 2.8)8	6.1(3.0- 11.5)7	10.8(5.8- 16.9)7
Kidney	0.8(0.3- 0.6)7	1.3(0.2- 3.3)9	7.2(3.5- 11.6)8	11.3(6.7- 15.3)6
Liver	0.2(0.1- 0.4)8	0.9(0.2- 1.4)7	4.9(1.1- 10.0)8	9.0(4.8- 10.7)6
Spleen	0.8(0.3- 1.5)6	2.0(0.5- 4.0)8	8.5(6.1- 11.1)8	6.7(4.3- 9.2)7
Heart	0.6(0.4- 0.7)7	1.8(0.5- 3.8)10	6.5(3.0- 10.7)8	8.6(5.8- 11)7
Muscle (Skeletal)	0.8(0.3- 1.7)7	1.7(0.3- 3.3)9	3.8(0.6- 7.2)8	5.3(3.1- 7.6)5
Stomach	1.3(0.7- 1.9)8	1.8(0.4- 3.1)9	5.8(2.4- 7.7)8	5.2(2.9- 7.6)6
Small Intestine	0.7(0.2- 1.2)7	1.6(0 - 2.6)8	9.6(4.7- 10.7)7	6.2(3.1- 9.3)7
Caecum	0.9(0.5- 1.2)6	2.2(1.2- 4.4)9	3.9(2.2- 7.1)8	4.0(3.3- 5.1)6
Large Intestine	1.0(0.4- 1.4)7	3.1(0.7- 5.7)9	5.5(2.6- 6.9)7	3.7(3.2- 4.3)5
Brain	0.9(0.3- 1.4)7	2.4(0 - 3.0)9	2.5(1.5- 3.0)8	3.7(1.6- 5.5)5
Fat	0.6(0.3- 0.9)8	1.7(0.5- 3.0)8	2.1(1.5- 3.9)8	2.4(1.7- 3.4)4
Testes	0.3(0.1- 0.5)8	1.3(0 - 3.7)10	3.0(1.9- 4.2)8	4.7(4.2- 5.4)6

*Data presented as average $\mu g\ F^-/gm$ tissue wet weight. The range of values is shown in parentheses, with the number of animals samples following.

TABLE XVII

Fluoride Concentration of Tissues of Normal Rats
(Group A was sacrificed and analyzed two months prior to Group B)

	A $\mu gF^-/gm$*	B $\mu gF^-/gm$
Bone	144 (112 -174)5	145 (125 -184)5
Lung	0.2(0 - 0.6)5	1.2(0.9- 1.7)5
Kidney	0.3(0 - 0.7)4	1.6(0.4- 3.2)5
Liver	0.3(0 - 0.6)5	0.7(0.3- 1.5)5
Spleen	1.0(0.4- 1.2)5	2.7(1.7- 6.9)5
Heart	0.7(0.4- 1.3)5	2.2(0.6- 4.7)5
Muscle (skeletal)	0.2(0 - 0.4)5	1.1(0.4- 2.3)5
Stomach	1.0(0.3- 1.5)5	2.7(1.8- 3.7)5
Small Intestine	0.3(0.2- 0.3)5	0.9(0.7- 1.2)5
Caecum	0.9(0.7- 1.2)5	2.2(0.5- 3.5)5
Large Intestine	0.7(0.6- 0.9)5	2.5(1.4- 3.7)5
Brain	0.4(0 - 0.8)5	0.8(0.5- 1.4)5
Fat	0.5(0 - 0.7)5	0.8(0.4- 1.8)5
Testes	0	0

*Data presented as average $\mu gF^-/gm$ tissue wet weight. The range of values is shown in parentheses, with the number of animals sampled following.

Reproducibility within each of the two latter groups is good, particularly when the potential sensitivity of the analysis, and the apparently inherent variability of fluoride deposition are considered. In the first series, however, the values observed are substantially less than in the second. This difference is apparently due to changes in dietary fluoride intake since the response of the analytical procedure and reagents could be demonstrated as constant by reference to fluoride standards.

The result of this fluctuation is that the overall fluoride distribution resulting from intoxication cannot be resolved beyond the limits of this difference unless elaborate control determinations are made with each experiment. Fortunately, the nature of the experiments is such that differences at this level in intoxicated animals cannot be considered biologically significant, and for this reason the time-consuming process of controlling each individual experimental determination can be avoided.

Distribution of Fluoride in Tissues of Rats Intoxicated with Sodium Fluoride and Inorganic Fluoride Oxidizing Agents.

The distribution in tissues of fluoride administered as sub-lethal doses of sodium fluoride has been used as a reference in the study of fluoride distribution in animals intoxicated with other agents. In this way, the behavior of fluoride originating with the inorganic fluoride oxidizing agents may be compared with movement and deposition of fluoride known to be present as fluoride ion.

No effort to examine the fluoride accumulation in the adrenal or thyroid glands was made. Because of the small size of the adrenal, it is doubtful that any difference in fluoride content could be detected which might be related to physiological changes. Thyroid tissue, on the other hand, should be examined after intoxication with NF_3 and N_2F_4 in future experiments, since Lee (ref. 41) has found that rats intoxicated with trifluoroamine oxide (NF_3O) accumulate fluoride in the thyroid. These observations were not considered essential to the current program, however.

The general distribution of fluoride in tissues of animals intoxicated with OF_2 was not investigated. Each exposed rat contacts only about 15 microliters (0.67 μmoles) of OF_2 in a ten-minute exposure at 15 ppm. This represents about 25 μg fluoride which cannot be measured in a 300 gram rat already containing sizeable amounts of fluoride.

Sodium Fluoride

In the period shortly after administration, the fluoride burden of a number of organs was greater in NaF-treated animals (Table XVIII) than in animals subjected to the other intoxicants, with the exception of BrF_5. This was probably due to the ready uptake of NaF from the site of injection followed by distribution at high concentrations in tissues. The distribution of NaF did not seem to reflect extensive movement of fluoride into the portal circulation from the intraperitoneal site of injection; this should presumably be manifested in high levels of fluoride in the liver. Plasma fluoride content, as expected, was high initially and decreased to normal ranges within 6 hours. The modest initial amount of fluoride found in red blood cells also soon decreased.

A transient rise of bone fluoride was seen in NaF treated normal animals; this tissue did not seem to be appreciably affected by other experimental agents.

Nitrogen Trifluoride

A generalized moderate increase in tissue fluoride appeared at the end of NF_3 inhalation and disappeared in one day (Table XIX). Departures from this regular pattern were apparent, however. High concentrations of fluoride in the red blood cells persisted through the sampling period, and possibly associated with the persistence of fluoride in the erythrocytes was the appearance of appreciable amounts of fluoride in the spleens of some animals during the 48 hours after exposure.

Tetrafluorohydrazine

The pattern of fluoride distribution after N_2F_4 treatment was similar to that seen after NF_3 exposure. The erythrocyte fluoride concentration was uniformly very high at a time when the general distribution in tissues was only moderately increased (Table XX). By two hours post exposure, however, most of the fluoride which had accumulated in tissues and plasma was mobilized. It is possible that the high plasma levels represented fluoride being transported to the kidney and to bone. Erythrocyte fluoride content was reduced to about 2/3 of its initial value after intoxication in 2 hours and then persisted at that level for a day or more. As in intoxication with NF_3, the spleen seems to serve to some extent as a fluoride reservoir, either in stored intact cells or from cells undergoing lysis.

TABLE XVIII. Distribution of Fluoride in Rat Tissues Following Intraperitoneal Injection of 30 mg NaF/kg

	Hours After Exposure			
	0.5	2	6	20
	μg F^-/gm*	μg F^-/gm	μg F^-/gm	μg F^-/gm
Plasma	18.4(5.0- 30)5	5.6(3.0- 11)5	1.0(1.0- 1.0)4	1.3(1.0- 2.0)4
R. B. C.	9.0(2.0- 15)5	3.1(2.0- 4.5)5	2.2(1.0- 5.0)5	1.6(0.5- 4.0)5
Bone	335.4(259 -382)5	243.0(181 -361)5	251.0(220 -311)4	284.0(241 -323)4
Lung	6.8(3.5- 10)5	5.2(3.0- 10.5)5	2.4(1.0- 4.5)5	2.9(1.1.0- 5.5)5
Kidney	13.2(6.0- 19)5	7.2(2.5- 11)5	4.7(2.0- 8.0)5	5.0(2.5- 8.5)5
Liver	11.0(2.70 15)5	2.6(1.0- 4.5)5	1.6(1.0- 2.0)5	1.5(1.0- 2.0)5
Spleen	15.2(6.0- 23)5	4.8(3.5- 7.0)5	2.4(1.5- 3.0)5	3.6(3.0- 4.0)5
Heart	7.8(4.0- 10)5	3.7(2.0- 5.5)5	2.0(n1.0- 3.0)5	3.5(2.0- 6.0)5
Muscle (skeletal)	4.4(1.0- 6.0)5	8.3(5.0- 13)5	2.4(0.5- 8.0)5	1.0(0.5- 1.5)5
Stomach	9.6(4.0- 13)	3.2(2.0- 5.5)5	1.8(0.5- 2.5)5	3.0(2.5- 4.5)5
Small Intestine	11.6(5.5- 20)5	4.8(3.0- 7.5)5	3.0(1.0- 6.0)5	2.3(1.5- 4.0)4
Caecum	4.8(4.0- 6.0)5	4.0(2.5- 6.5)5	2.7(2.0- 3.5)5	3.0(1.5- 4.0)4
Large Intestine	9.1(7.5- 10)3	2.2(1.5- 4.0)4	4.5(1.0- 8.0)5	2.7(1.0- 4.0)3
Brain	2.7(2.0- 4.5)3	2.0(0.5- 3.0)3	4.1(1.0- 9.5)5	3.2(1.0- 6.0)5
Fat	5.3(3.5- 7.5)5	2.2(1.0- 5.0)5	2.7(1.0- 5.0)5	3.2(1.0- 5.0)5
Testes	12.4(6.0- 31)5	2.6(2.0- 3.5)5	1.5(1.0- 2.0)5	2.0(1.0- 3.5)5

*Data presented as average μg F^-/gm tissue wet weight. The range of values is shown in parentheses, with the number of animals sampled following.

TABLE XIX

Distribution of Fluoride in Rat Tissues Following Exposure to 5,000 ppm NF_3 for 48 Minutes

	Hours After Exposure		
	0	2	6
	µg F^-/gm	µg F^-/gm	µg F^-/gm
Plasma	4.6(5.8- 16.0)3		
R. B. C.	23. (18.5- 30.0)3	15.1(14.9- 15.4)2	24.9(18.2- 33.0)3
Bone	198 (178 -227)3	197 (140 -239)3	250 (185 -294)3
Lung	3.0(2.5- 3.5)3	2.0(0.7- 3.5)3	2.4(1.2- 3.9)3
Kidney	6.9(5.7- 8.5)3	3.5(2.3- 5.7)3	4.5(1.2- 9.8)3
Liver	2.6(1.6- 3.3)3	0.9(0.6- 1.3)3	0.9(0.7- 1.3)3
Spleen	5.7(1.0- 11.2)3	10.1(2.8- 23.4)3	12.3(3.7- 28.6)3
Heart	5.4(3.7- 7.5)3	3.5(1.5- 6.5)3	3.8(1.8- 5.9)3
Muscle (skeletal)	2.2(1.6- 3.0)3	2 (0.7- 4.1)3	2.8(0.3- 4.8)3
Stomach	2.1(0.8- 2.6)3	1.7(1.3- 2.3)3	3.2(1.2- 6.5)3
Small Intestine	1.4(1.0- 1.8)2	2.2(1.7- 2.9)3	1.1(0.1- 2.3)3
Caecum	4.9(3.3- 8.0)3	3.6(2.0- 6.7)3	3.5(2.5- 5.6)3
Large Intestine	2.9(1.9- 4.3)3	3 (2.2- 5.0)3	1.7(1.1- 2.3)2
Brain	2.9(3.2- 3.7)2	1.5(0.1- 1.9)3	1.6(0.4- 3.6)3
Fat	2.4(1.2- 4.1)3	2.3(1.3- 3.2)3	2.3(1.7- 2.4)3
Testes	4.3(2.6- 5.5)3	3.6(1.9- 6.6)3	2.2(0.5- 5.5)3

	12	24	48
R.B.C.	18.8(18.4- 19.3)2	23.6(17.7- 31.7)3	16.4(14 - 18.8)3
Bone	324 (251 -318)	253 (251 -318)3	243 (207 -263)3
Lung	1.8(1.6- 2.0)3	2.5(2.2- 3.0)3	1.5(1.5- 1.5)2
Kidney	2.2(1.9- 2.5)3	1.8(1.7- 2.0)3	1.2(1.1- 1.4)2
Liver	3.1(2.9- 3.5)3	1.1(0.5- 2.0)3	0.7(0.7- 0.8)2
Spleen	4.4(1.7- 6.0)3	4.3(1.5- 5.8)3	6.1(5.8- 6.5)2
Heart	3.4(2.0- 5.1)3	2.2(2.2- 2.3)3	4.1(2.7- 5.6)2
Muscle (skeletal)	0.7(0.7- 0.8)3	0.7(0.5- 0.8)3	0.7(0.5- 1.0)2
Stomach	1.9(1.6- 2.5)3	1.6(1.2- 2.2)3	1.3(0.8- 1.8)2
Small Intestine	1.6(0.7- 2.9)3	1.2(1.1- 1.3)3	0.6(0.4- 0.9)2
Caecum	3.6(2.3- 5.0)3	3.4(2.3- 5.5)3	1 (0.9- 1.2)2
Large Intestine	1.9(1.2- 2.7)3	3.2(2.2- 3.7)	1.6(1.1- 2.2)2
Brain	1.0(0.8- 1.2)3	0.8(0.7- 0.9)3	0.8(0.8- 0.8)2
Fat	1.2(1.1- 1.2)3	1.4(1.2- 1.8)3	1.1(1.1- 1.2)2
Testes	1.3(1,0- 1.5)3	1.4(0.9- 2.1)3	2.1(1.2- 3.1)2

TABLE XX. Distribution of Fluoride in Rat Tissues Following Exposure to 10,000 ppm N_2F_4 for 15 minutes

	Hours After Exposure			
	0	2	6	20
	$\mu gF^-/gm$*	$\mu gF^-/gm$	$\mu gF^-/gm$	$\mu gF^-/gm$
Plasma	13.4(9.7- 17.9)6	1.6(0.8- 2.6)4	1.3(0.4- 1.9)3	2.9(0.9- 9.3)6
R. B. C.	30.3(23.9- 33.5)6	23.0(17.4- 31.7)3	20.5(19.4- 21.6)2	16.7(6.1- 24.6)7
Bone	241 (183 -280)4	218 (157 -279)2	316 (293 -343)3	278 (229 -326)4
Lung	9.8(8.0- 11.0)3	5.5(3.6- 7.5)4	4.0(3.5- 4.5)3	3.2(2.0- 5.4)7
Kidney	9.7(6.5- 12.0)3	4.9(2.9- 9.7)4	1.7(1.0- 2.5)3	2.8(1.2- 10.0)7
Liver	7.0(6.5- 7.5)3	2.0(1.7- 2.4)4	1.4(0.7- 2.0)3	1.4(1.1- 4.4)7
Spleen	9.1(7.4- 11.0)3	5.0(3.5- 7.1)4	3.2(2.0- 4.0)3	5.7(3.6- 7.3)7
Heart	8.6(7.0- 11.3)3	3.1(1.1- 5.9)4	2.7(2.0- 3.5)3	2.8(1.2- 4.0)7
Muscle (Skeletal)	4.3(3.0- 5.3)3	1.5(1.4- 1.6)3	0.9(0.6- 1.5)3	1.1(0.4- 2.0)6
Stomach	7.6(4.7- 10.0)3	3.2(1.9- 3.8)4	2.7(2.0- 3.0)3	1.8(1.5- 2.6)5
Small Intestine	4.7(2.5- 7.0)2	2.4(2.2- 2.7)3	1.7(0.7- 2.5)3	2.4(1.5- 3.7)7
Caecum	6.7(5.0- 9.0)3	2.5(1.1- 3.8)4	3.3(2.0- 4.0)3	3.2(2.0- 4.9)7
Large Intestine	5.7(4.5- 6.5)3	3.7(3.0- 3.8)4	2.2(1.0- 3.5)3	3.6(2.1- 5.7)7
Brain	2.4(1.5- 3.7)3	1.4(1.0- 1.6)4	3.3(1.5- 4.5)3	1.5(0.6- 3.7)7
Fat	2.0(1.0- 3.6)3	1.6(0.7- 2.2)4	2.1(1.5- 3.0)3	1.3(0.7- 2.0)7
Testes		1.4(1.2- 1.8)4	1.0(0.7- 1.5)3	1.0(0.4- 1.6)7

*Data presented as average $\mu gF^-/gm$ tissue wet weight. The range of values is shown in parentheses, with the number of animals sampled following.

Chlorine Trifluoride and Bromine Pentafluoride

Intoxication of rats by the interhalogens results in a uniform pattern of fluoride distribution, but large quantitative differences are apparent when effects of the two agents are compared. Fluoride from inhaled BrF_5 deposited at relatively high levels in most tissues, which persist for several hours (Table XXI). The fluoride originating as inhaled ClF_3 is found at lower concentrations; there is some evidence that the spleen is able to concentrate fluoride from ClF_3, and at the second hour after exposure a questionable increase in lung fluoride also appears (Table XXII).

Excretion of Fluoride Into the Digestive Tract by Rats Intoxicated with NaF, Interhalogens and NF_3

An investigation of fluoride levels in the visceral contents was made after preliminary observations indicated that excretion of fluoride into the digestive tract of animals intoxicated with interhalogens might differ from that observed in sodium fluoride experiments.

The most interesting findings of this series of experiments do not relate directly to the behavior of toxic inorganic fluorides. We have found that the concentration of fluoride in the contents of the hollow organs of normal rats on a standard diet is remarkably constant from animal to animal (Table XXIII), despite substantial differences in total visceral content. Since the fluoride concentration of visceral contents of normal rats has relatively little variance from animal to animal, it may be applied as a correction in estimating the concentration and amount of fluoride transported into the digestive tract after intoxication. It is also clear that fluoride ion is excreted into the lumen of the gastrointestinal tract in significant quantities. These facts are closely related in terms of the present problem and are of independent physiological significance as well.

That substantial amounts of fluoride arrived in the digestive tracts of intoxicated animals by a route other than ingestion is vividly shown in the case of the interhalogens; concentrations of fluoride ion were extremely high in the visceral contents of BrF_5 treated animals, and substantial in those intoxicated by ClF_3 (Table XXIV). The animals used for the BrF_5 studies, however, were apparently removed from their cages just prior to their feeding period, resulting in very limited amounts of ingesta in the hollow organs. This has resulted in concentrations of fluoride seemingly excessive, although the absolute amount of fluoride found is not extreme. No control measurements have been made of the fluoride content of fasted animals.

TABLE XXI. Distribution of Fluoride in Rat Tissues Following Exposure to 500 ppm BrF_5 for 30 Minutes

	Hours After Exposure			
	0	2	6	20
	μg F⁻/gm	μg F⁻/gm	μg F⁻/gm	μg F⁻/gm
Plasma	5.8(4.5- 7.0)6	4.5(3.5- 5.5)5	3.0(2.0- 4.5)5	1.7(1.0- 2.5)4
R. B. C.	3.3(1.5- 5.5)6	3.2(2.0- 4.5)5	2.3(1.5- 3.0)5	2.1(0.5- 35)4
Bone	300 (235 -378)6	319 (284 -349)4	367 (256 -493)5	353 (278 -418)4
Lung	5.9(3.0- 8.5)6	7.3(2.5- 15.0)5	3.6(2.5- 6.5)5	2.0(1.5- 3.0)4
Kidney	5.8(4.5- 7.0)5	5.9(3.5- 10.1)5	5.0(4.5- 5.5)5	2.6(2.0- 3.0)4
Liver	4.5(3.5- 6.0)6	4.1(2.5- 6.0)4	4.4(2.0- 8.5)5	1.7(1.5- 2.0)4
Spleen	5.1(3.5- 8.0)6	6.3(4.0- 10.0)5	5.4(3.0- 7.5)5	3.3(2.0- 5.5)3
Heart	4.4(3.5- 5.5)6	4.6(3.0- 6.0)4	3.0(2.0- 4.0)5	2.1(1.0- 4.0)4
Muscle (skeletal)	3.7(2.5- 5.0)5	5.0(2.0- 7.0)5	3.4(3.0- 4.0)5	2.4(2.0- 3.0)4
Stomach	15.0(14.0- 15.5)3	8.7(2.1- 16)3	8.3(6.5- 12)3	5.4(4.8- 6.3)3
Small Intestine	6.5(4.9- 9.0)3	6.0(2.6- 9.2)3	5.6(4.7- 7.2)3	6.0(3.8- 7.4)3
Caecum	6.2(5.5- 6.5)3	4.3(1.9- 7.0)3	4.5(3.4- 6.4)3	4.2(1.8- 5.6)3
Large Intestine	6.0(3.1- 10.8)3	4.4(2.2- 6.1)3	4.5(3.5- 6.4)3	6.9(4.5- 9.2)3

*Data presented as average μg F⁻/gm tissue wet weight. The range of values is shown in parentheses, with the number of animals sampled following.

TABLE XXII. Distribution of Fluoride in Rat Tissues Following Exposure to 400 ppm ClF_3 for 15 Minutes

	Hours After Exposure			
	0	2	6	24
	μg F^-/gm	μg F^-/gm	μg F^-/gm	μg F^-/gm
Bone	118 (97 -135)4	140 (106 -177)5	140 (106 -178)5	172 (128 -232)5
Lung	2.6(1.4- 4.2)4	4.0(0.9- 7.3)5	1.2(0.7- 1.9)5	1.3(0.2- 3.2)4
Kidney	1.9(1.0- 2.4)4	2.4(1.8- 2.9)4	1.3(0.7- 2.2)5	1.6(0.4- 3.7)5
Liver	1.7(1.1- 2.3)4	1.4(0.8- 2.6)4	0.7(0.3- 1.6)5	1.1(0.4- 2.1)3
Spleen	6.5(1.6- 12.0)4	5.3(1.2- 9.8)5	2.4(1.0- 4.7)4	1.4(0.8- 2.0)3
Heart	4.4(1.4- 7.1)4	1.9(1.4- 2.6)4	1.2(0.4- 2.7)5	1.3(0 - 2.6)5
Muscle (skeletal)	2.9(1.1- 4.3)4	1.4(0.6- 2.2)5	1.3(0.8- 2.0)4	1.0(0.6- 2.0)4
Stomach	3.1(0.6- 8.3)3	2.7(2.1- 3.7)3	1.8(0 - 3.8)3	3.4(2.7- 3.9)3
Small Intestine	1.3(1.2- 1.4)3	4.3(2.6- 6.6)3	2.5(1.7- 3.3)3	2 (1.6- 2.3)3
Caecum	1.5(0 - 1.1)3	1.5(0.8- 2.2)3	5.4(1.7- 12)3	3.7(1.9- 5.5)3
Large Intestine	1.7(1.0- 2.1)3	2.5(2.2- 3)3	3.6(2.7- 4.3)3	3.1(3.0- 3.2)3
Brain		2.9(1.5- 5.5)4	1.9(0.7- 3.1)5	1.2(0.4- 2.4)5
Fat	2.3(0.6- 4.8)4	2.9(1.3- 4.9)5	1.6(0.7- 2.6)5	1.5(0.7- 2.0)5
Testes	2.2(1.2- 2.8)4	3.9(0.8- 8.5)5	2.4(0.8- 5.1)5	1.3(0.8- 1.8)5

*Data presented as average μg F^-/gm tissue wet weight. The range of values is shown in parentheses with the number of animals samples following.

TABLE XXIII

Fluoride Concentration of Visceral Contents of Normal Rats Fed a Standard Laboratory Diet

	Stomach	Small Intestine	Caecum	Large Intestine
Average μg F^-/gm dry weight	42.4	55.8	137.3	161.6
Variance	15.3	64.8	82.7	200.5
Standard deviation	3.9	8.0	9.1	14.1
Number of animals	9	11	9	10

The average total fluoride of gastro-intestinal contents from ClF_3 treated animals increased very rapidly in the first two hours; in BrF_5 treated rats, these values continued to increase for 48 hours (Table XXIV). This probably does not stem from any fluoride deposition and release phenomenon in the lung. The lung, as the tissue of contact, at no time had a fluoride burden significantly in excess of other tissues, in spite of its immense surface to mass ratio. A reasonable explanation in view of the demonstrable immediate reactions of interhalogens in aqueous systems is that substantial amounts of fluoride ion were released into the circulation at the time of exposure, and were temporarily taken up in tissues to be released during the succeeding few hours. This behavior closely resembles that of NaF.

The greater tissue fluoride burden demonstrated in animals exposed to BrF_5 may be explained by the much higher quantity of fluoride contacted by these animals. If a ventilation rate of 100 milliliters per minute is assumed for the rats intoxicated by inhalation in these experiments, it can be calculated that exposure to ClF_3 resulted in contact with about 1.5 mg of fluoride ion and that about 6.3 mg were introduced as BrF_5. These figures may be compared with estimates that rats intoxicated with NaF contacted 4.5 mg fluoride ion and that rats exposed to OF_2 only contacted 25 μg.

During NF_3 intoxication, rats contacted more than 100 mg of fluoride, a much greater amount than was introduced in any other form. Apparently only a small fraction of this amount became absorbed and there was clearly no large residual pool of fluoride to emerge over succeeding hours.

The peak of fluoride excretion into the digestive tract was at the end of exposure, which corresponds with the peak of methemoglobin concentration. This is probably not accidental. The physical properties and reactivity of NF_3 suggest that turnover of NF_3 to fluoride should be during its participation in the hemoglobin-methemoglobin cycle and possibly, to a very minor extent, with other reducing systems with which its extremely low solubility might allow contact. At the end of exposure, the source of NF_3 was terminated and the only residue other than that found in erythrocytes was the limited amount of fluoride in transit from these cells. The red blood cells were probably the source of the slight increases in gastro-intestinal fluoride content which appeared during the 24 hours after exposure. Six hours after exposure, erythrocytes may contain more labile fluoride than the entire remainder of the body, with the possible exception of the spleen.

We have suggested that NF_3 enters the body primarily as a function of its reaction with hemoglobin. Using the rate of entry of NF_3 related to methemoglobin formation which has been observed in dogs, it is reasonable to estimate that between 2,000 and 3,000 micrograms of fluoride was transported across the lung of each rat during the NF_3 exposures used in these experiments. About 4-5% of this amount was found in the digestive tract at the end of the exposure.

Fluoride introduced as sodium fluoride was apparently moved into the upper digestive tract and into bone after absorption from the peritoneal space, and then slowly returned to the circulation for disposibion in urine. With the exception of the initial sampling, the average amounts found in the large intestine are within the range found in normal animals, indicating that reabsorption and excretion keep pace with release of fluoride from tissue.

The possibility that fluoride enters the digestive tract in saliva must be considered in the case of each agent. In animals treated with sodium fluoride, for example, there is no proof, after the second hour of observations, that fluoride is not added to the visceral content by passage down the digestive tract. However, in three of the five animals examined 30 minutes after injection of sodium fluoride, gastric fluoride was negligible, and the small intestine of two of these animals contained high levels. Caecal fluoride was likewise high in some animals.

TABLE XXIV. Concentration of Fluoride as Average μg/gram Dry Weight of Visceral Contents of Rats Following Intoxication with Sodium Fluoride and with Inorganic Fluoride Oxidizing Agents

	Hours After Exposure		
	0 $\mu gF^-/gm$	2 $\mu gF^-/gm$	6 $\mu gF^-/gm$
NaF 30 mg/kg, intraperitoneally			
Stomach	81(0- 204)5	94(39- 204)4	44(22- 60)4
Small Intestine	164(88- 175)5	187(116- 248)4	68(34- 128)4
Caecum	183(88- 232)5	208(140- 282)4	225(186- 293)4
Large Intestine	.221(159- 320)5	144(12- 214)4	206(188- 226)4
NF_3 5,000 ppm, 48 minute exposure			
Stomach	212(102- 398)3	53(25- 79)3	
Small Intestine	278(91- 638)3	130(104- 157)3	
Caecum	164(160- 171)3	158(130- 180)3	
Large Intestine	143(129- 163)3	145(124- 162)3	
ClF_3 400 ppm, 15 minute exposure			
Stomach	183(20- 442)3	196(149- 244)3	362(72- 116)3
Small Intestine	94(82- 109)3	351(190- 617)3	451(204- 827)3
Caecum	118(107- 141)3	129(118- 149)3	192(161- 217)3
Large Intestine	139(122- 155)3	139(138- 141)3	169(90- 267)3
BrF_5 500 ppm, 30 minute exposure			
Stomach	2866(1971-4152)3	770(488- 914)3	1329(1099-1505)3
Small Intestine	106(65- 147)2	1033(880-1121)3	686(541- 879)3
Caecum	174(444- 105)3	194(182- 206)3	435(69- 639)3
Large Intestine	112(75- 183)3	155( )1	600(286- 914)2

Hours After Exposure			
12 µgF⁻/gm	20 µgF⁻/gm	24 µgF⁻/gm	48 µgF⁻/gm
	50(34- 70)6 42(6- 98)6 208(184- 236)6 96(31- 170)6		
58(38- 86)3 94(57- 140)3 166(146- 205)3 167(145- 205)3		44(33- 76)3 49(48- 85)3 215(198- 229)3 227(189- 256)3	92(56- 121)3 20(16- 25)3 153(92- 251)3 316(101- 690)3
113(45- 242)3 47(14- 103)3 367(356- 374)3 413(378- 448)3		77(21- 108)3 154(110- 211)3 360(281- 412)3 415(391- 460)3	146(0- 254)3 58(21- 86)3 275(86- 448)3 333(317- 357)3
1187(583-1507)3 288(104- 454)3 932(681-1337)3 1737(881-3073)3		847(489-1396)3 303(33- 678)3 495(113- 800)3 652(460- 761)3	386(54- 719)2 290(60- 685)3 979(800-1260)3 954(696-1153)3

Bell, et al., in studying the fate of the sodium salt of fluoride-18 in cattle was able to determine that fluoride excretion in saliva was relatively low. In his work, 500 milligrams of sodium fluoride were administered, and output of fluoride in saliva did not exceed 1.3×10^{-4} % of the administered dose per milliliter of saliva (ref. 42). An average output of less than 1×10^{-4} % per milliliter may be assumed by inspection of the data in the report. If a salivary secretion rate in excess of 50 liters daily is accepted as normal for the bovine (ref. 43), the output of fluoride in saliva reaches only 1.2% of the administered dose during the first 6 hours after administration. A similar low salivary secretion of 0.08% of administered fluoride-18 in 21 minutes was shown by Wills (ref. 44) who demonstrated as well that the ratio of salivary to plasma chloride was much higher than the ratio of salivary to plasma fluoride. Carlson has also shown that salivary fluoride is much lower in concentration than the fluoride found in plasma (ref. 45).

In each of the series of experiments in this laboratory, the amount of fluoride found in the gastro-intestinal tract immediately after exposure represents a much greater percentage of the administered dose than was seen in any of the above quoted experiments and it is doubtful that a significant fraction of the gastro-intestinal fluoride is of salivary origin.

The possibility of serosal to mucosal transport of sodium fluoride across the visceral wall should be considered, since the material was originally placed in the peritoneal cavity and such activity in vitro has been described by Parkins (ref. 46). The generalized extensive disposition of fluoride in organs, however, indicates that NaF is taken up by the circulation before moving into the visceral contents.

In any biological system in which there are extensive storage mechanisms or regulatory systems, substantial variability should be expected when extra loading is imposed. In contrast to the constancy of fluoride concentration seen in the organs of normal rats, those of intoxicated rats often contained highly variable concentrations of fluoride, occasionally including amounts much lower than those of control animals. One explanation may be that the flux across the gut wall is variable or cyclic and dependent upon regulations other than the concentration gradient.

The response by intoxicated animals to extraneous fluoride is somewhat unique. Most systemically distributed chemicals are subjected to limited storage and rapid excretion when introduced into an animal.

There is reason to believe that fluoride is subject to ready storage and limited excretory activity. We have found that fluoride in urine of rats appears to reach an equilibrium concentration that is relatively constant regardless of the amount of fluoride available for disposition. This tentative conclusion has been only partially verified in our experiments, but interpretation of data presented by Wallace (ref. 47), indicated that urinary fluoride-18 excretion at higher levels of intake is markedly reduced when compared with the excretion of trace amounts.

Resistance to excretion is probably responsible for the persistence of fluoride in the tissues of BrF_5-treated animals, which contacted a much larger amount of fluoride than those intoxicated with ClF_3. The amounts retained by animals injected with NaF are probably explained by the same reasoning, since they each received a total of about 4.5 mg of fluoride ion, which is approximately 3 times the amount contacted during ClF_3 exposure, and substantially more than the fluoride actually available to NF_3 treated animals.

In view of these influences upon the distribution of fluoride ion, there is nothing in the evidence reported here that might indicate the existence and deposition of a degradation product of the interhalogens or NF_3 more complex than fluoride ion.

SECTION V

CONCLUSIONS

At the completion of the present program, it is possible to suggest, with reasonable confidence, the general nature of the acute effects of each of the agents we have studied. The interhalogens apparently cause direct corrosive damage to the pulmonary surface, with resultant failure of gas exchange. NF_3 and N_2F_4 cause methemoglobin formation to an extent considered lethal, per se, probably by different but potentially related mechanisms. There is, as well, evidence of additional undefined pharmacologic effects of the nitrogen fluorides or their products. OF_2 appears to enter the pulmonary cells as a non-ionized gas, perhaps to react there with and inactivate some essential mechanism readily susceptible to oxidation or to release HF within the cell which in turn may interfere specifically with some metabolic process. The result is delayed pulmonary cellular destruction and death.

The research program described in this report had the common objective of all toxicologic study--the protection of personnel who inadvertently contact the agents. This goal has been approached, but not yet achieved. Ideally, this process should result in effective antidotal therapy applicable to any intoxicated organism. Practically, we have found that the mechanism of the interhalogens is such that no therapy can be effective because of destruction of essential tissues. The only protection is therefore absolute isolation from the toxic material. The nitrogen fluorides are much less destructive and are of such limited and non-residual effect that infrequent short term low exposures may ultimately be considered tolerable.

The single compound for which the entire cycle of determination of mechanism and subsequent development of an antidote must be carried through is OF_2. This agent is one of the most lethal of all chemicals. It is in a sense a systemically acting intoxicant, even though it apparently does not reach the circulation. Because it appears to act within pulmonary cells, it is possible that an antagonist might be developed which can interfere with the presumed intracellular oxidation. Depending on the potential deployment of this agent, it appears that further establishment of the mechanism, and progress toward a protective and therapeutic treatment is of critical importance.

APPENDIX

LIST OF MATERIALS, INSTRUMENTS, EQUIPMENT AND THEIR SOURCES

1. Chlorine trifluoride, bromine pentafluoride, nitrogen trifluoride, tetrafluorohydrazine, nitric oxide, nitrosyl fluoride. Air Products and Chemicals, Allentown, Pennsylvania.

2. Oxygen difluoride. General Chemical Division of Allied Chemical Company, New York.

3. Nitrogen dioxide and sulfur hexafluoride. The Matheson Company, East Rutherford, New Jersey.

4. Drierite. W. A. Hammond Drierite Company, Xenia, Ohio.

5. Teflon (DuPont trademark) stock, all shapes. Pennsylvania Fluorocarbon Company, Inc., Philadelphia, Pennsylvania.

6. Kel-F #90 grease. Minnesota Mining and Manufacturing Company, St. Paul, Minnesota.

7. I^{125} RISA (radioiodinated serum albumin). Abbott Laboratories, North Chicago, Illinois.

8. Silver chloride crystals for infrared cell windows. Harshaw Chemical Company, Cleveland, Ohio.

9. Swagelok Tube Fittings. Crawford Fitting Company, Salem, Ohio.

10. Valves (Teflon 2-way, 3-way) sizes of Teflon tubing fitted with female luer. Hamilton Company, Whittier, California.

11. Corning Model 12 pH meter with calomel reference electrode #476002 and Ag/Agcl triple purpose electrode #476022. Corning Glass Works, Corning, New York.

12. Delmar Portable high vacuum system. Delmar Scientific Laboratories, Inc., Maywood, Illinois.

13. Beckman IR-5A Infrared Spectrophotometer. Beckman Instruments, Fullerton, California.

14. Beckman D-B Spectrophotometer. Beckman Instruments, Fullerton, California.

15. Beckman Recorder. Beckman Instruments, Fullerton, California

16. Peristaltic pump, model T6SH. Sigmamotor Company, Middleport, New York.

17. Centrifuge Model L-708 with microhematocrit head. Phillips-Drucker, Astoria, Oregon.

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13. ABSTRACT

Studies have been made of the toxicology and chemistry of nitrogen trifluoride, tetrafluorohydrazine, chlorine trifluoride, bromine pentafluoride, and oxygen difluoride. Lethality of each agent by inhalation has been determined, as well as estimates of lethality after intraperitoneal administration. Experimental evidence suggests that the lethal effect of interhalogens is by corrosive local destruction of pulmonary surfaces, resulting in failure of gas exchange. Oxygen difluoride is thought to pass intact into the pulmonary cells where it reacts with biochemical reducing systems to ultimately cause cell death and structural failure. The nitrogen fluorides both cause intrinsically lethal levels of methemoglobin formation, but other pharmacologic activity by these compounds or their derivatives may also contribute to their toxic activity.

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Bromine pentafluoride, BrF_5						
Oxygen difluoride, OF_2						
Inhalation toxicology						
Fluoride in tissues						
Hemoglobin reactions						